Ameliorative mechanism of dietary components on cadmium or mercury-induced toxicities in PC12 cells

(PC12 細胞におけるカドミウムまたは水銀によって引き起こされる毒性に対する 食物成分の改善メカニズム)

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Abbreviations

ALA/LA	α-lipoic acid
BBB	Blood brain barrier
BHT	Butylated hydroxytoluene
Cd	Cadmium
DHLA	Dihydrolipoic acid
DNA	Deoxyribonucleic acid
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
Hg	Mercury
LDH	Lactate dehydrogenase
MT	Metallothionein
mTOR	Mammalian Target of Rapamycin
Nrf2	Nuclear factor erythroid 2-related factor 2
ROS	Reactive Oxygen Species
Se	Selenium
SOD	Superoxide dismutases
Zn	Zinc

Abstract

Environmental pollution is one of the greatest challenges faced by the global society and continues to be a world concern. Pollution caused by toxic metals receives considerable attention due to the impacts they introduce to the environment and human health. Metals such as mercury (Hg), cadmium (Cd), arsenic (As), lead (Pb), nickel (Ni), chromium (Cr) and cobalt (Co) are highly toxic even in minor quantity. Recently contaminations of metals in food stuffs and associated health risks have been reported extensively and led interest of searching for medicines of metals toxicity. In this regard, dietary components can be appropriate option and offer great potentials of being therapeutics against metals, with least side effects. In this study, essential micronutrients selenium (Se) and zinc (Zn), dietary supplement dihydrolipoic acid (DHLA) and food additive butylated hydroxytoluene (BHT) have been used to evaluate the protective actions against metals-induced harmful effects in PC12 cells, which is a model cell line for molecular toxicity assessment. In this research it

was hypothesized that “food components have regulatory effects on metals mediated toxicity at cellular level”.

In chapter 2, Se was used to detoxify Cd in PC12 cells in a concurrent exposure. The purpose was to investigate the mechanism/s of cyto-protection by Se (Na_2SeO_3; Se^{4+}) against Cd ($CdCl_2$; Cd^{2+})-induced cytotoxicity. In addition, Se (5, 10, 20 and 40 μM) and Cd (2.5, 5 and 10 μM)-induced cytotoxicity was determined. Cytotoxicity assays and western blot analyses confirmed that Se (≥10 μM) promotes autophagic cell death via inhibition of mTOR activation and p62 accumulation due to increase of cellular oxidative stress. On the other hand, co-presence of non-toxic Se (5 μM) and toxic Cd (5 μM) was showen to increase cell viability, glutathione and glutathione peroxidase 1 (GPx1) levels, and to decrease DNA fragmentation and lactate dehydrogenase (LDH) activity compared to Cd-treated (5 μM) cells alone. Furthermore, western blot analyses of cytochrome c and ERK1 indicated that Cd-induced apoptotic cell death in PC12 cells. However, the co-exposure of Se with Cd significantly decreases the release of cytochrome c into cytosol from mitochondria, and up-regulates ERK1 protein to inhibit Cd-induced apoptosis. In conclusion, Se (≥10 μM) possess cytotoxicity in PC12 cells; however, co-presence of Se (5 μM) with Cd (5 μM) protects against Cd-induced apoptosis in PC12 cells due to inhibition of Cd-induced oxidative stress and subsequently suppression of mitochondrial apoptosis pathway.

Another extremely dangerous environmental contaminant is Hg, that responsible for human diseases including neurological disorders. However, the mechanisms of inorganic Hg （iHg**)**-induced cell death and toxicity are little known. DHLA is the reduced form of a naturally occurring compound lipoic acid, which act as a potent antioxidant through multiple mechanisms. In chapter 3, it was hypothesized that DHLA has an inhibitory role on iHg-cytotoxicity. The purposes of this research were to investigate mechanism/s of cytotoxicity of iHg, as well as, the cyto-protection of DHLA against iHg induced toxicity using PC12 cells. Treatment of PC12 cells with $HgCl_2$ (Hg^{2+}) (0-2.5 μM) for 48 h resulted in significant toxic effects, such as, cell viability loss, high level of lactate dehydrogenase (LDH) release, DNA damage, cellular glutathione (GSH) level decrease and increased Hg accumulation. In addition, protein level expressions of akt, p-akt, mTOR, GR, NFkB, ERK1, Nrf2 and HO-1 in cells were downregulated; and cleaved caspase 3 and cytochrome c release were upregulated after Hg^{2+} (2.5 μM) exposure and thus inducing apoptosis. However, pretreatment with DHLA (50 μM) for 3 h before Hg^{2+} (2.5 μM) exposure showed inhibition against iHg^{2+}-induced cytotoxicity by reversing cell viability loss, LDH release, DNA damage, GSH

decrease and inhibiting Hg accumulation. Moreover, DHLA pretreatment reversed the protein level expressions of akt, p-akt, mTOR, GR, NFkB, ERK1, Nrf2, HO-1, cleaved caspase 3 and cytochrome c. So, it can be concluded that DHLA could attenuate Hg^{2+}-induced cytotoxicity through boosting up of antioxidant defense, limiting Hg accumulation and inhibition of apoptosis in cells.

In chapter 4, the ameliorations of Se counter to iHg-mediated toxicity in PC12 cells was investigated in simultaneous exposure. Cytotoxicity assays have been shown that iHg (5 μM) encouraged oxidative stress and intrinsic apoptosis *via* oxidizing glutathione, damaging DNA, degrading cell membrane integrity, down-regulating mTOR, p-mTOR, akt and ERK1, and up-regulating cleaved caspase 3 and cytochrome c release in PC12 cells 48 h after incubation. Again, co-treatment of Se (5 μM) appeared to inhibit intrinsic apoptosis and oxidative stress induced by iHg (5 μM) *via* boosting GPx contents, improving reduced form glutathione contents, limiting DNA degradation, improving cell membrane integrity, up-regulating mTOR, p-mTOR, akt, ERK1 and caspase 3, and down-regulating cleaved caspase 3 and cytochrome c leakage in PC12 cells. In addition, flow cytometry analysis confirmed that apoptosis induced by iHg toxicity was improved by Se-co-exposure. The contribution of nrf2 and HO-1 activation was not significant in iHg-toxicity or Se-protection mechanisms in PC12 cells. In conclusion, these results recommended that glutathione level decrease acts a critical role in iHg influenced oxidative stress and co-treatment of Se attenuates iHg-cytotoxicity through its antioxidant properties.

In chapter 5, the regulatory role of BHT was evaluated against inorganic Hg-induced cytotoxicity by a co-treatment method in PC12 cells. BHT is a phenolic component generally consumed as a food additive as an antioxidant. However, the cellular mechanisms of BHT induced antioxidant properties against heavy metals-influenced toxicity are little studied. It was hypothesized that BHT has a regulatory effect on Hg induced cytotoxicity. The aim of this research was to assess the protecting effects of BHT against iHg-toxicity in PC12 cells, where cells were treated with/without $HgCl_2$ (Hg^{2+}) (5 μM) and BHT (100 μM) for 48 h and analyzed further. Cells treated by iHg caused a significant reduction of cell viability, cell membrane damage, glutathione reduction, DNA degradation. Moreover, iHg treatment caused suppressed expressions of p-akt, akt, mTOR, ERK1, nrf2, HO-1, bcl-xL, caspase 7 and caspase 3 expressions; and elevated expressions of p53, bax, cytochrome c and active caspase 3. However, BHT and Hg^{2+} co-exposure showed prevention against iHg^{2+}-influenced toxicity by improving cell viability, LDH release, DNA damage and GSH content. Additionally, BHT co-treatment inverted the expressions of akt, p-akt, mTOR, ERK1, Nrf2,

HO-1, caspase 7, caspase 3, cleaved caspase 3 and cytochrome c. These findings proved that BHT inhibits Hg^{2+}-mediated toxicity *via* enhancing antioxidant defense, and hindering toxicity and intrinsic apoptosis.

In chapter 6, the effect of Zn on inorganic Hg-induced cytotoxicity in the PC12 cells were investigated. Zinc (Zn) is an essential micronutrient with a wide range of antioxidant properties. The cells were treated with $HgCl_2$ (5 µM) for 48h with/without 1 h prior $ZnCl_2$-treatment (100 µM) and deliberated for further analysis. After 48 h of incubation with only Hg^{2+}, the cell showed reduced cell viability, compromised cell membrane, increased DNA degradation, depleted glutathione level, and drastically increased apoptotic cells. Subsequently, Hg^{2+}-treated cells demonstrated a significant downregulation of akt, mTOR, ERK1, nrf2, HO1, bcl2, bcl-xL, and upregulation of p53, bax, cytochrome c and cleaved caspase 3, indicating intrinsic apoptosis induction. However, cells pretreated with Zn^{2+} before Hg^{2+}-exposure showed a significant improvement in cell viability, cell membrane, DNA damage, glutathione level and apoptotic cells, with a significant upregulation in mTOR, akt, p-akt, ERK1, nrf2, HO1, bcl2 and bcl-xL, and downregulation in p53, bax, cytochrome c and cleaved caspase 3, indicating inhibition of intrinsic apoptosis by Zn^{2+}-pretreatment. The findings suggested that Zn^{2+}-pretreatment not only improves glutathione content but also induces activation of nrf2-HO1 pathway, which would tend to suppress mercury cytotoxicity and inhibit intrinsic apoptosis induced by Hg.

Heavy metals (Cd and Hg)-exposure even in trace amount can cause deleterious health effects and diseases in human. However, these diet components (Se, DHLA, BHT and Zn) have proper ameliorative actions against metal toxicity. This research recommended that Se, DHLA, BHT and Zn could be used as therapeutic agents in combating metal induced effects in biological systems and suggested further study in animal models for better understanding.

Chapter 1: General introduction

1.1. Background

Environment has a major role on its biological system as well as on human health. Environmental pollutants such as toxic metals and organic substances are major public health concern because of their potential for induction of diseases. Some trace metals from environment are essential for human body, e.g., zinc (Zn), copper (Cu), selenium (Se), chromium (Cr), iron (Fe), cobalt (Co), manganese (Mn), and molybdenum (Mo). The doses of deficiencies and toxicity are different for each of them (Nodeberg et al., 2000). Whereas, some metals are recognized as toxic, e.g., lead (Pb), arsenic (As), mercury (Hg), cadmium (Cd) which are bound with other substabces in environments. Metal extraction and purification from the nature has been the major cause of metal contaminants in high concentrations to human and all living beings. Industrial waste discharge (containing heavy metals) without adequate treatment is another major cause of metal pollution in environment, and these metals eventually accumulated into food chain. Metals exposure to human can be caused from food, water, occupational environment, house hold environment (cooking instruments and cutlery) and personal care products. These toxic metals are accumulated and deposited in brain, bones, kidney and liver in humans (Haouem et al., 2007; Glomski et al., 1971; Antonini et al., 2009; Barregård et al., 1999). Researchers also reported that fetus is at great risk when pregnant mother is exposed to high level of toxic metals especially organic metals and showed severe toxicity (Bowring, 2005; Riess and Halm, 2007; Wang et al., 2009). The effects of toxic metal exposure on unborn were illustrated in stem cells of cord blood by autophagy and human spermatozoa immobilization (Di Gioacchino et al., 2008; Holland and White, 1982).

Metal exposure can cause a long risk of physiological disorders including developmental defect, mental retardation, cognitive impairment, Parkinson's and Alzheimer's diseases, sclerosis Autism spectrum disorder (ASD) and so on (Bjorklund et al., 2017). Metal toxicity may impose structural and functional alterations and damages in different organs, e.g., nervous system (Bakar et al., 2017), cardiovascular system (Takahashi et al., 2017), renal system (Li et al., 2015) and lungs (Hirano, 1996). Exposure to heavy metals, especially Hg and Cd has been considered to cause many pathological disorders. Major sources of Hg exposure can be included dental amalgam, sea foods, vaccines, energy saving lights and thermometer and cosmetics (Bjorklund et al., 2017; Rahman et al., 2018); whereas Cd sources of exposures are smoking, mining, smelting and refining of nonferrous metals, fossil

fuel combustion, incineration of Cd-containing waste, production of phosphate fertilizers, recycling, electric and electronic waste (Rahman et al., 2018). Hg and Cd sources, route of exposure, and effects in human health have been listed in **Table 1.1** (Rahman et al., 2018).

Table 1.1: Hg and Cd sources, route of exposure, and effects in human health (Rahman et al., 2018).

Metal(-oids)	Sources of exposure	Route of exposure	Tolerable limit	Health effects	References
Cd	Smoking, mining, smelting and refining of nonferrous metals, fossil fuel combustion, incineration of Cd-containing waste, phosphate fertilizers production, recycling, electric and electronic waste etc	Ingestion, inhalation and dermal contact.	7 µg/kg body weight/ week	Pulmonary edema, respiratory diseases, renal dysfunction, anemia, osteoporosis, cancer, neurological disorder, olfactory dysfunction; learning disability, hyperactivity and birth defects	IPCS (2005-2007), WHO (2010)
Hg	Mining, gold extraction, batteries, thermometers and barometers, electric switches, light bulbs, dental amalgam, cosmetics, pharmaceuticals	Inhalation, ingestion and dermal contact.	1.6 µg/kg body weight/week.	Neurological and behavioral disorders, tremors, insomnia, memory loss, neuromuscular effects, headaches and cognitive and motor dysfunction, kidney and immune effects, neurodevelopmental problems in the developing fetus.	WHO (2007), EPA (2017)

Numerous studies have been operated to investigate the toxic effects manifestation and the toxicokinetics of heavy metals on biological systems using animal and cellular models. A brief description about the toxicokinetics will be presented in later parts of this chapter.

Deleterious effects of toxic metals /heavy metals on health will lead to discover a solution/remediation for combating these situations. Dietary components can often be a better solution with least side effects to detoxify the biological system from heavy metals. In this chapter, it will be described the toxic effects of heavy metals in biological systems and roles of the potential food components (Se, DHLA, BHT and Zn) in detoxification of toxic metals on biological systems. In addition, at the end of this chapter the motivation and aims of this research will be discussed including food components as medical roles.

1.2. Cd as a toxic heavy metal

Cd is classified as Group I carcinogen to humans by International Agency for Research on Cancer (IARC, 1993). It is a widely used heavy metal with a large varieties of sources for human exposure such as volcanic activity, weathering and erosion, and river transport; along with various anthropogenic sources such as smoking, mining, smelting and non-ferrous metals refining, fossil fuel combustion, incineration of municipal waste (batteries and plastics with Cd), phosphate fertilizers production, and recycling electric and electronic waste (UNEP, 2008; WHO, 2010). The major human exposure routes for Cd are through inhalation or ingestion. Based on particle size, 10-50% of inhaled Cd dust is absorbed (Bernhoft, 2013). Dermal absorption is relatively insignificant. Exposure to Cd occurs from ingestion of contaminated foods (e.g., crustaceans, meats, leafy vegetables, rice) or contaminated water, and can cause long-term health effects. Absorption of Cd in intestine is larger in Fe, Ca, or Zn-deficient persons (Nordberg et al., 2007). Cigarette smoking is reflected as the most significant source of Cd exposure. Cd amounts in blood and kidney are consistently higher in smokers compared to the non-smokers (Friberg, L. 1983). According to direction of WHO (2010), the tolerable monthly intake rate for Cd is 25 μg/kg body weights, whereas 3 μg/L for drinking water and 5 ng/m^3 for the air (annual average). The Biological limit values (BLVs) of Cd for blood and urine are defined as 1 μg/L and 0.8 μg/L, respectively (Hartwig et al., 2017).

Cd accumulation is responsible for respiratory tract irritation, pulmonary edema, anemia, renal dysfunction, osteoporosis, cancer, birth defect, neurological disorder, learning disability and hyper activity in children (Chen et al., 2008a; Lau et al., 2006; Rahman et al., 2019). Furthermore, Cd can cause neurobehavioral defects and olfactory dysfunction in Cd-exposed

workers (Lau et al., 2006). It was reported that Parkinson's disease and Alzheimer's disease are linked to Cd (Chen et al., 2008b, 2008a). Cd has been accumulated vastly through the intestinal mucosa, and transfers at a low rate by diffusive transfer mechanism into the organism (Elsenhans et al., 1997). After absorption, Cd is majorly accumulated in the liver and kidneys, particularly in the renal cortex (Jadan-Piedra et al., 2018).

Fe, Zn, dietary fiber, proteins and probiotics amounts in the body affect intestinal absorption of Cd. The key intestinal transporter of Fe, the functional divalent metal transporter 1 (DMT1) protein, is upregulated in the small intestine by body Fe depletion and this upregulation elevate Cd uptake from the gastrointestinal tract (Park et al., 2002). A study on the US adults stated that dietary and serum Zn are connected to Cd exposure, and affects Cd absorption and accumulation. A 10% increase of serum Zn level caused a 2% decrease in blood Cd and a 4% increase in urinary Cd levels (Vance and Chun, 2015). An *in vivo* study on rat demonstrated that fiber-rich wholegrain wheat containing regular diet lowered Cd bioavailability, and an increased dietary Zn intake depressed Cd absorption and retention (House et al. 2003). The effect of proteins on gastrointestinal Cd-absorption appeared to depend on type of proteins and the length of the treatment. Mice fed with high-protein diets for 24 h before and after oral administration of Cd showed lower Cd amounts in the liver, kidney and whole body; whereas, in long-term exposures the high protein diet caused high amounts of Cd absorption in tissue (Revis and Osborne, 1984). Kojima et al. (1985) showed that glycine and ovalbumin proteins significantly decreased gastrointestinal absorption of Cd; though gelatin did not. Zhai et al. (2013; 2014) demonstrated that a probiotic *Lactobacillus plantarum* CCFM8610 decreased the intestinal absorption and accumulation of Cd in the liver and kidneys.

1.3. Toxicity mechanisms of Cd and disease manifestation

In different countries daily intake of Cd from foods is at the level of 10-35 μg per person (WHO 2011). Cd can induce toxicity to the cell through interfacing with receptors on the cell surface even without entering the cell (Beyersmann and Hechtenberg 1997). Cd binds to the receptors and prevents their proper functions. Cd forms bonds (covalent and ionic) with the sulfhydryl groups, disulfide, carboxyl, imidazole or multiple amino compounds in the cells, triggering significant imbalance in their homeostasis (Bertin and Averback, 2006). Cd may interfere with cell signaling pathways at every stage of signal transduction, and can act on receptors, second messengers, transcription factors (Jing et al., 2012). The main target organelle of Cd is the mitochondria (Koizumi et al., 1996). Cd enters into the mitochondria

via Ca-channels, and causes conformational changes in the membrane proteins through binding to thiol groups. As a result, Cd alters the oxidative phosphorylation, and disrupts its membrane permeability initiating membrane potential reduction, ATP levels drop, imbalance in homeostasis of Ca, Na, K, eventually release of cytochromes, Fe (II) ions causing reactive oxygen species (ROS) production and a lot of implications (Sarkar et al., 2013). Those implications include excess amounts of ROS formation and alteration in gene expressions which initiate cell cycle arrest, differentiation, immortalization, and apoptosis. Several studies reported apoptosis mediated by Cd-toxicity in different organs; however, non-apoptotic cell death is also induced (Cuypers et al., 2010).

Waisberg et al. (2003) reported that carcinogenic action due to Cd may implicate interference of intercellular signaling and cytoskeleton destruction with altered cell adhesion. These reactions also include modification of E-cadherin and β-catenin, which is accountable for tissue integrity. Cd replaces calcium in E-cadherin, causing conformational changes in the protein. As results, Cd eventually disrupts intercellular connections, and leads to β-catenin activation and unrestrained proliferation, resulting apoptosis and interrupts or encourages cancer development. Impaired signaling by Cd can also affect transcriptional and translational level. Cd causes increased concentration of Ca ions that activate phosphorylation of cAMP response element binding protein (CREB) to bind specific sites in the promoter region of gene for gene expression (Hechtenberg, et al., 1996).

From the above, it has been considered that Cd is deeply involved in gene expression. First, metals and metalloids cause cancer according to direct interaction with DNA. Due to low mutagenicity, Cd was considered to work as an epigenetic or indirect genotoxic carcinogen (Waalkes and Misra, 1996). In addition, Cd may act on the gene expression by deterring DNA methylation (Takiguchi et al., 2003). Overexpression and excessive synthesis of protein syntheses in hypomethylation are accountable for cell proliferation increases which may cause malignancy development. Several researches have informed that Cd induced alterations in DNA methylation levels (Benbrahim-Tallaa et al., 2007; Huang et al., 2008; Joseph, 2009).

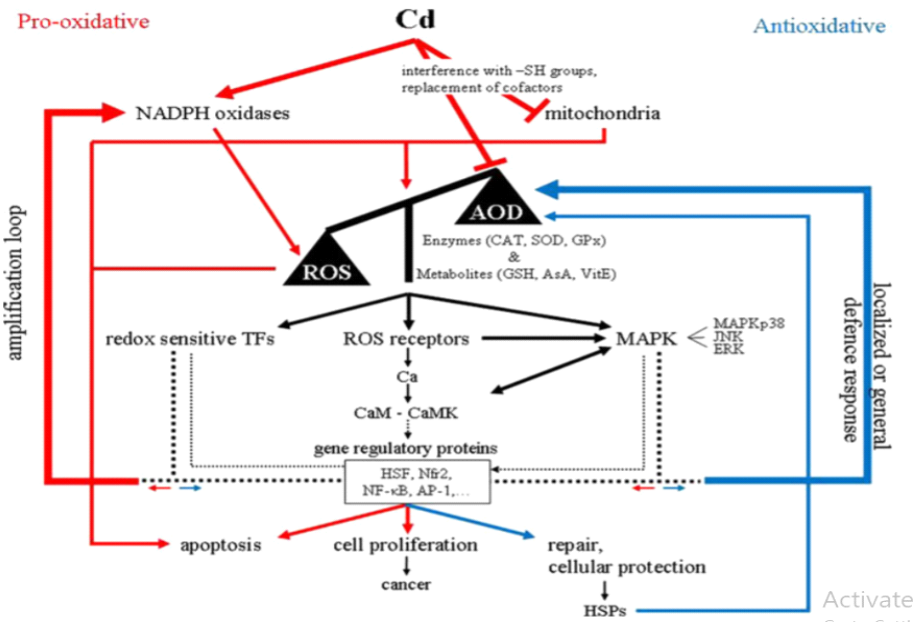

Figure 1.1: Redox-related pathways during cadmium stress. Cd induces oxidative stress via indirect pathways such as through the induction of NADPH oxidases, via binding with thiol groups and by replacing Fenton metals from their active site. A disturbed redox balance influences both damaging (apoptosis and uncontrolled cell proliferation) as well as repair processes, via the activation of several signaling cascades. Both MAPK and Ca-dependent signaling pathways are important during Cd stress, although exact interactions are still not known. AP-1: activation protein-1; AsA: ascorbic acid; CaM: calmodulin; CaMK: calmodulin kinase; CAT: catalase; ERK: Extracellular Signal-Regulated Kinase; GSH: glutathione; HSF: heat shock factor; HSP: heat shock protein; JNK: Jun N-terminal Kinase; MAPK: Mitogen-Activated Protein Kinase; NF-jB: nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2: nuclear factor erythroid 2 related factor 2; TF: transcription factor; VitE: vitamin E (Cuypers et al., 2010).

Cd has been reported to influence over-expression of Early Response Genes (IEGs) (c -fos, c-jun and c-myc) in different cell lines such as rat L6 myoblasts (Jin and Ringertz, 1990), rat kidney NRK-4 9 cells (Von Zglinicki et al., 1992), mouse embryonic fibroblasts (Joseph et al., 2001), rat and human mesangial cells (Wang and Templeton, 1998) and human prostate epithelial cells (Achanzaret al., 2000). Again, stress-response genes are involved in the synthesis of MT, genes encoding heat shock proteins, genes related to glutathione (GSH - the most important antioxidant in cells) synthesis and homeostasis, and genes related to oxidative stress responses. It is believed that protein denaturation and damage induced by Cd stimulates the gene inductions encoding heat shock proteins (Morimoto et al., 1994). The Cd-induced

testicular toxicity was associated with a lack of MT gene induction in the sertoli and spermatogenic cells in rats, indicating a protective role for MT in Cd-induced toxicity (Xu, 2003). GSH and its enzymes such as GSH peroxidase (GPx) and GSH reductase (GR) act a central part in Cd-detoxification. γ-glutamyl cysteine synthetase which enhances GSH synthesis has played an important role of underlying the GSH-mediated protection from Cd-carcinogenesis (Singhal et al., 1987). Recent studies also indicated that Cd may affect the transcription and translation processes (Sarkar et al., 2013). Fig 1.1 also showed a hypothesized depiction of redox-related pathways during Cd stress.

1.4. Hg as a toxic heavy metal

Hg is a persistent toxic heavy metal, and has been designated as one of the ten most dangerous chemicals to public health by WHO. Hg is recognized as neurotoxic and immunotoxic and induces great risk to human and environmental health because of its natural and anthropogenic sources. The halflife of inorganic Hg is several years to several decades in human brains (Bjørklund et al., 2017). Hg in the environment is under different chemical forms such as elemental Hg (metallic), inorganic Hg and organic Hg (Bjørklund et al., 2017a). The exposure routes of Hg are inhalation, ingestion and dermal contact. Hg in elemental form (Hg^0) has been released from thermostats, thermometers, dental amalgams, and latex paint (Patrick, 2002) to the atmosphere resulting occupational health risk and Hg is bio-absorbed by inhalation. Organic Hg is well known to be able to easily across the blood-brain barrier (BBB) (Clarkson and Magos, 2006). Inorganic Hg (Hg salts; iHg) originates from laxatives, cosmetic products, teething powders, diuretics and antiseptics. Organic Hg forms are majorly methyl mercury (MeHg), and ethyl mercury (EtHg). They come from mainly sea foods and are the most hazardous of Hg species (Crowe et al., 2017).

Intake of fish and shellfish during more than 30 days is considered as cause of 75% of blood Hg (Mahaffey et al., 2004). Syversen and Kaur (2012) showed that by inhalation of Hg-vapor 80% of MeHg was absorbed, while by oral exposure about 100% of MeHg was absorbed. All forms of Hg after being bio-adsorbed are converted to Hg^{2+} (Clarkson and Magos, 2006).

WHO (2007) indicated that for pregnant women the provisional tolerable intake of MeHg is 1.6 μg/kg bodyweight per week to protect the fetus from neurotoxic effects. According to WHO guidelines, recommended concentration for total Hg are 1 μg/L and 1 $\mu g/m^3$ (annual average) for water and air, respectively. The organic Hg absorbed (80%) is higher than inorganic Hg absorbed (JECFA, 2011). Absorption of Hg^{2+} is inconstant and relies on the solubility of Hg salts (JECFA, 2011). Because of the high bonding affinity between Hg and

sulfur and along with the interactions between mercuric ions and the thiol group(s) in proteins, peptides and amino acids, most of the inorganic Hg accumulated in kidney, especially the pars recta of the proximal tubule (Zalups, 2000). However, organic Hg is highly accumulated in kidneys and liver, and excreted (80-90%) through feces in the form of inorganic Hg. Several studies have indicated a relationship between organic-Hg exposure and increased risks of neurodevelopmental disorders such as tic disorder, attention-deficit/hyperactivity disorder (ADHD) and delayed language skills (Hviid et al., 2003; Young et al., 2008). Hg may cause immunological alterations (Berlin et al., 2015).

1.5. Toxicity mechanisms of Hg and disease manifestation

Toxic action caused by Hg can be taken through various mechanisms in the body. Molecular and cellular studies of organic Hg in the nervous system have suggested that Hg^{2+} may play a major role as it is resulted from the conversion of EtHg or MeHg. The presence of Hg^{2+} in neurons results from conversion of organic Hg in glial cells (Hargreaves et al., 1985; Tiffany-Castiglioni and Qian, 2001). Furthermore, the levels of Hg^{2+} after EtHg exposure were found higher than those after MeHg exposure, whereas only after MeHg exposure damaged granular layer was observed. Thus, it was suggested that the demethylation action or Hg^{2+} formation could not be the major responsible factor for MeHg neurotoxicity (Magos et al., 1985). It was also proposed that demethylation of MeHg happened in glial cells and then Hg was moved to neurons, eventually caused the MeHg neurotoxicity (Syversen and Kaur, 2012). Also, both CH_3Hg^+ and Hg^{2+} demonstrate strong affinity to thiol (-SH) groups, and play an important role in the toxic mechanism of Hg (Risher and Tucker, 2017).

Various subcellular components including the membrane systems involve free thiol groups for their proper functions. Different forms of Hg can attack thiol groups in the proteins or membranes, and when Hg reacts with the sulfur amino acid residues in membranes or proteins, the physiological and metabolic functions could be diminished or blocked (Ynalvez et al., 2016). Furthermore, oxidative stress (Garg and Chang, 2006; Yin et al., 2007), impaired Ca homeostasis (Dreiem and Seegal, 2007), and the glutamate homeostasis alterations (Farina et al., 2003; Yin et al., 2007) have been stated to be involved in the sub-cellular neurotoxicity of MeHg. Existing reports showed that there are some substantial similarities between the neurotoxic mechanisms of MeHg, EtHg and elemental or inorganic Hg, though there are some differences in metabolic rates of MeHg and EtHg. These considerations were summarized in a recent review by Risher and Tucker (2017).

1.5.1. Interaction with sulfhydryl groups

Hg compounds react with thiol groups in neuroglia and neurons. The neurotoxic effects of mercurial are facilitated by the capacity of blocking essential thiol groups in cellular enzymes and membranes (Syversen and Kaur, 2012). Neuronal microtubules which form the neuronal cytoskeleton, are essential components for intracellular transport (Bjørklund et al., 2017a) It could be attacked by mercurials (Syversen and Kaur, 2012). In addition, neuroinflammation, including microglial activation could be induced by Hg compounds (Dewi et al., 2012). Cellular SH-levels and their localizations can be the cause of the selective toxicity of Hg compounds (EFSA, 2012).

Low molecular weight thiol, cysteine, plays a vital role in the circulation and transport of Hg compounds through the body. Thus, MeHg-cysteine mimic as the essential amino acid, methionine and MeHg was imported into cells by the same mechanism. Then again, chelating thiols such as dimethylcysteine (penicillamine), meso−2,3 dimercaptosuccinic acid (DMSA) and sodium 2,3-dimercaptopropanesulfonate (DMPS) impede uptake of mercurials in to the cells (Aaseth et al., 1981). DMSA and DMPS are considered as drugs for treatment of Hg toxicity (Rooney, 2007; Aaseth et al., 2016a). Chelating agents such as alpha-lipoic acid (ALA), a disulfide, and its metabolite dihydrolipoic acid (DHLA), the corresponding dithiol, are reported to have chelation properties against Hg (Ou et al., 1995).

Different than MeHg, elemental Hg vapor (Hg^0) is taken up by diffusion into neurons and subsequently oxidized to Hg^{2+}. Hg^{2+} reacts with thiol groups in most proteins and tripeptide GSH (Clarkson, 2002). Sulfur containing amino acids supplements such as taurine, cysteine, and methionine are recognized to be effective in metal toxicity mitigation (Agha et al., 2014). These supplements can contribute the excretion and reduction of associated oxidative stresses.

1.5.2. Autoimmunity and immune activation by Hg exposure

Autoimmune diseases can be caused by an interface between genetic susceptibility and different factors (such as gender, ethnicity, age, and environment), which encourage diseases (Crowe et al., 2017). Environmental factors such as medicines and toxic metals including Pb, Au and Hg could induce autoimmune diseases in animal models, and in occupationally exposed humans (Jha et al., 2014). All studied salts of Hg have immune modulatory prospective and allergenic characteristics (Stejskal et al., 1996). Hg has been reported to arouse autoimmunity in genetically susceptible animals (Stejskal, 2015a) and can stimulate the progress of autoimmunity in humans (Prochazkova et al., 2004). On the other hand, Hg-containing compounds can persuade immunomodulation, immunosuppression,

immunostimulation, delayed-type hypersensitivity and autoimmunity, through altering the immune cytokines production (Kern et al., 2014; Berlin et al., 2015).

Toxic metal, Hg can act as a hapten, creating a complex with biological macromolecules, which together act as an antigen (Vas and Monestier, 2008; Kern et al., 2014). Autoimmune diseases in Hg exposed animals with lupus like syndrome and over-production of specific autoantibody, were reported (Silbergeld et al., 2005). MeHg exposed mice also suffered from autoimmunity (Crowe et al., 2017). In addition, clinical experiments showed that Hg exposure could induce multiple sclerosis and other autoimmune diseases (Stejskal, 2015a). Besides, 72% of patients in a study with oral lichen planus (autoimmune disease) showed a significant response to Hg *in vitro* (Stejskal et al., 1996) and after removal of dental amalgams, local and systemic symptoms remarkably decreased.

1.5.3. Effects on DNA, RNA and protein synthesis

MeHg interaction with DNA, RNA and protein synthesis has been reported in numerous studies (Syversen and Kaur, 2012; Basu et al., 2014). It is suggested that the binding of Hg to SH-groups has a major role in secondary changes in DNA and RNA and structural modifications in ribosomal proteins. Hg exposure may be related with epigenetic changes (e.g. DNA methylation) (Goodrich et al., 2013). Hg exposure can be associated with DNA hypomethylation in brain tissue of the polar bear (Richard Pilsner et al., 2010). Hg exposure has a role in the hypermethylation of Rnd2 gene in Hg-treated mouse embryonic stem cells (Yoshikazu et al., 2011). Many researchers reported that protein synthesis has been reducedby Hg exposure both *in vivo* and *in vitro* (Yoshino et al., 1966; Cavanagh and Chen, 1971; Syversen, 1981). The nhibition of protein synthesis by iHg was reported approximately ten times higher than that by MeHg in the cell-free systems prepared from mouse glioma, rabbit reticulocytes and Yoshida ascites sarcoma cells (Nakada et al., 1980).

1.5.4. Interaction of mercury with microtubules

Microtubules are filamentous intracellular structures which are responsible for cellular structure and movements in all eukaryotic cells. Different kind of cellular functions such as axonal and dendritic transport, neuronal growth and differentiation structural maintenance and cellular migration are depending on the microtubules (Bjørklund et al., 2017). The depolymerization and derangement of cerebral microtubules can be caused by MeHg because of high affinity of it for sulfhydryl groups (SH-groups) of tubulin (Carratù and Signorile, 2015). Also, MeHg-exposure could be a potential risk factor for neuropsychiatric and

neurodegenerative diseases in neurons. It induces discrepancies in hippocampus dependent spatial learning and memory during adolescence (Tian et al., 2016). MeHg can cause damaging effects on cytoskeletal proteins (microtubules) and cytoskeleton-regulating proteins, initiating troubles in neuronal migration and differentiation (dos Santos et al., 2016).

1.5.5. Interaction of mercury with membrane transport system

The cellular uptake of Hg can be divided into active, energy-dependent (e.g. MeHg-cysteine) or passive (e.g. MeHgCl in cell cultures) depending on the Hg species (Heggland et al., 2009). Inhibition of the glutamine/amino acid (ASCT2) transporter, which has a significant role in clearing excytotoxic L-glutamate levels from the extracellular space, could be induced by MeHg (Oppedisano et al., 2010). In addition, chloride concentration and pH affect iHg (Hg^{2+}) diffusion through planar lipid bilayer membranes (Gutknecht, 1981). Moreover, simultaneous incubation with organic methylmercury chloride (MeHgCl) and thimerosal permitted Hg to cross the BBB in both directions. At that time, Hg was slightly higher accumulated in the brain facing compartment, whereas the BBB cells transfers Hg out of the brain for iHg, (Lohren et al., 2016).

MeHg is associated with sulfhydryl-containing molecules (e.g. cysteine) in the environment, to constitute a complex of MeHg‑cysteine (MeHg-S-Cys), as a mimic of the neutral amino acid, methionine which can be transported via the L-type neutral amino acid carrier transport (LAT) system. The effects of MeHg (provided as MeHg-S-Cys)-induced neurotoxicity in mice, and further investigations revealed that L-Met increases cerebellar Hg deposition in mice exposed to MeHg and simultaneous exposure to MeHg, and that L-Met could induce larger motor impairment than MeHg alone (Zimmermann et al., 2014).

1.5.6. Interactions of mercury with glutathione and cellular redox balance

The major thiol group, GSH provides protective effects as a cofactor for GPx, one of the selenoenzymes. Astrocytes, especially the cortical astrocytes of brain cells, can synthesize a high amount of GSH which can be the reason for their comparative resistance against Hg toxicity (Bjørklund et al., 2017a). In addition, GSH levels influence the uptake of iHg in brain tissue; a 20% decreases of GSH levels in brain lead to a considerable increase of Hg levels in brain (Eide and Syversen, 1983). Again, ROS formation and decreases of GSH are major mechanisms of MeHg-induced toxicity (Farina et al., 2011a). The MeHg-induced neurotoxicity was mediated by an imbalance between the oxidative and reductive cellular processes. After MeHg exposure reduced amounts of GSH synchronize with preeminent of

ROS contents (Stringari et al., 2008). Though, it was indicated that MeHg exposure could cause both an increase and a decrease in GSH levels (Grotto et al., 2010).

The medicinal herb, Bacopa (Brahmi) could normalize MeHg-induced reduction of GSH levels (Ayyathan et al., 2015). Moreover, the Amazon fruit, *Euterpe oleracea* extract provided a protective effect of MeHg toxicity, probably via antioxidative polyphenols (Brasil et al., 2016). Apocynin, an NADPH oxidase inhibitor and antioxidant substance, inhibits mitochondrial damage and oxidative stress induced by MeHg in cultured bovine aortic endothelial cells, indicating an involvement of NADPH oxidase in this toxicity (Ghizoni et al., 2017).

1.5.7. Interaction of mercury with metallothioneins

Metallothioneins (MTs) which are a family of cysteine-rich and low molecular-weight proteins with 61‑68 amino acids, are the most common intracellular metal-binding proteins due to their many SH groups (Aschner, 1996; Andrews, 2000; Malekzadeh and Shahpiri, 2017). The binding of Hg in MTs has been studied thoroughly due to their involvement in metal detoxification and free radicals scavenging (Krizkova et al., 2016). MTs could protect the brain and gastrointestinal tract over toxic metals overload. Metals such as Cd and Zn are strongly bound to MTs, and also potent inducers of apo-metallothionein synthesis (Bjørklund, 2013). Zn is the most abundant element and Cu is also very significant of the MT-inducing elements (Park et al., 2001). Some toxic metals such as Cd and Hg could cause substantial disturbance of Zn and Cu metabolism as they are MT inducers even in a small amount (Underwood, 1977). MT gene erasure could raise the susceptibility to Hg-induced neurocognitive impairment (Eddins et al., 2008). MTs were believed to be modulated various level of Hg neurotoxicity (Faber et al., 2009).

1.6. Overview of Se, DHLA, BHT, Zn antioxidants

1.6.1. Se

Se is an essential trace element with crucial physiological functions and is the most comprehensively investigated chemo-preventive compounds (Goldhaber, 2003; Thomson, 2004). Major sources of Se are bazil nut, broccoli, carrots, cabbage, garlic, mushrooms, cheese and other vegetables, meats, cereals, and grains which comprise of different forms and quantities of Se (Navarro-Alarcon and Cabrera-Vique, 2008). The recommended daily intake is 50 μg/day, where the toxic dose was estimated at 350‑700 μg/day (Council, 2000). Low intake of Se (<25 μg/day) can cause Keshan disease (a juvenile cardiomyopathy), which is

avoidable with Se supplements (Zeng, 2009). As a component of several vital selenoproteins, Se is essential for numerous cellular processes, even in cancer prevention (Lu and Holmgren, 2009; Papp et al., 2007). Selenocysteine (as a form of Se) is a component of 25 classes of selenoproteins, including GPxs, selenoproteins, P, W, and R, and thioredoxins (Tapiero et al., 2003; Diwadkar-Navsariwala, et al., 2004, 2006). Several of these selenoproteins showed antioxidant activities, while the purposes of most selenoproteins have not yet been discovered. In food, Se is in covalent forms including inorganic salts, amino acids, and methylated compounds; whereas in Se-supplements Se consists of the inorganic forms such as sodium selenite, sodium selenate or the organic forms such as selenomethionine (SeMet), Se-methylselenocysteine (SeMSC), and high-Se yeast (Ip et al., 2000; Schrauzer, 2001). In human Se is better absorbed and retained in the form of SeMet and Se-yeast than the inorganic Se forms (Finley, 2006). The organic sources contain Se in the reduced state (selenide: Se(-2)); however, in the inorganic salts Se remains in oxidized forms (selenite: Se(+4); selenate: Se(+6)). After absorption Se compounds are converted from the higher-valence forms to the selenide (Se(-2)) using reduced GSH and NADPH; however, the organic forms (SeMet, selenocysteine (SeCys)) converts Se in the selenide state via catabolism (Kieliszek and Błażejak, 2013).

Supplement of low doses of Se is not only used in cancer treatment, but also contribute in various cellular functions by limiting inflammations, heart diseases and blood pressure regulation (Brozmanova et al., 2010).

Se compounds are known as an antioxidant for its ROS scavenging ability. Takahashi et al. (2005) investigated the ROS scavenging ability of six selenocarbamates where all of them show superoxide scavenging ability. As a result, methyl-N-(4-methylphenyl) selenocarbamate and methyl-N-phenylselenocarbamate showed the highest radical scavenging activity. Takahashi et al. (2005b) also reported the superoxide scavenging activity of selenourea compounds. De Silva et al. (2004) reported that selenomethonine and several phenylamino selenoxides prevented 31‑40% of DNA damage mediated by peroxynitrite.

One of the 25 known classes of selenoproteins is GPxs, which act as antioxidants by neutralizing peroxides (such as H_2O_2). An efficient and well-known GPx mimic, ebselen was reported to protect biological molecules from oxidative damages. Li and Cao (2002) demonstrated that ebselen prevents dopamine/Cu^{2+}/H_2O_2-influenced DNA damage, through reducing H_2O_2 and radical scavenging. In Japan ebselen is also permitted in clinical treatment of stroke (Yamaguchi et al., 1998).

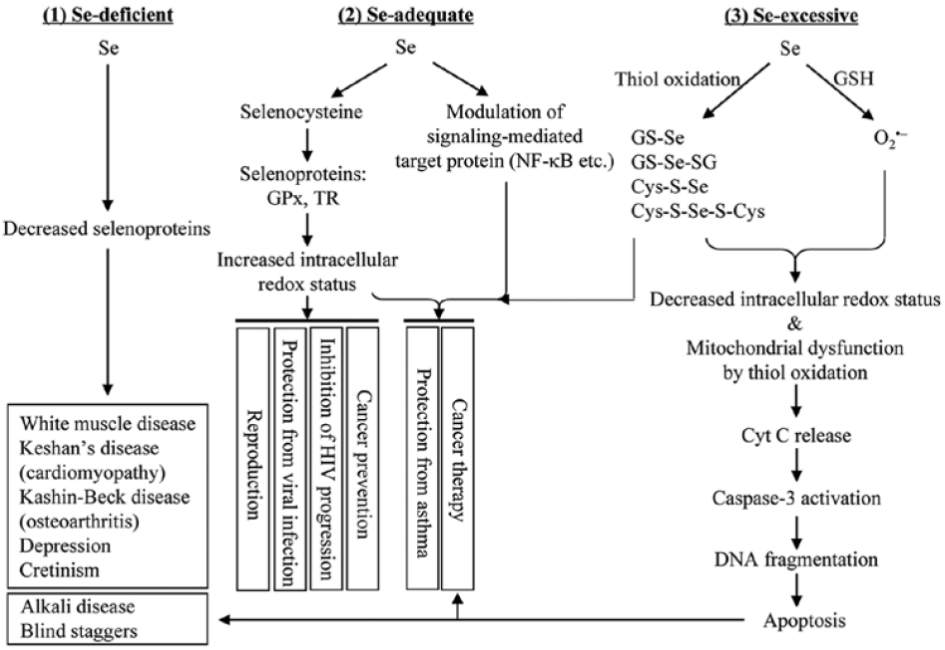

Figure 1.2: Various biological functions of various Se binding compounds. Several clinical symptoms caused by Se deficiency include white muscle disease, Keshan's disease (cardiomyopathy), Kashin-Beck disease (osteoarthritis), depression, and cretinism. Se at physiological levels plays an important role in the cellular redox regulation, existing as selenocysteine at the catalytic site of antioxidant enzymes and inducing intracellular redox potential and thiol modification of the targeted proteins such as NF-κB. High levels of selenium often cause selenium toxicity (apoptosis), which results from the generation of oxygen radicals, mitochondrial dysfunction and DNA damage. TR, thioredoxin reductase; GSH, reduced glutathione; Cyt C, cytochrome c (Lee and Jeong, 2012).

Metal-binding property also related to Se antioxidant activity. Therefore, metal-binding with Se-containing antioxidant substances and enzymes has been investigated by several researchers. Battin et al. (2011) reported that organic selenium compounds, selenomethionine, selenocystine, methyl-selenocysteine, and other compounds inhibit copper-mediated DNA damage. They also demonstrated methyl-selenocysteine, selenocystamine, 3,3-diselenobispropionic acid and 3,3-selenobispropionic acid prohibited iron-mediated DNA damage. They suggested that the antioxidant activity of these Se compounds with copper and iron was acted via metal-binding property which is different from enzyme activity such as GPx. As like organo-Se compounds, the antioxidant activity for inorganic Se compounds was recognized to be metal binding as the primary mechanism in preventing iron-induced DNA

damage (Ramoutar and Brumaghim, 2007). Fig 1.2 shows various biological functions provided by Se depending on its intake concentrations.

1.6.2. DHLA

Alpha-lipoic acid (LA) is a popular constituent in multivitamin formulas and anti-aging supplements, and well-known as a free radicals scavenger, metals chelator, and intracellular GSH restorer. α-LA (also called 1,2-dithiolane-3-pentanoic acid) is a naturally occurring compound enzymatically *de novo* synthesized from octanoic acid in the mitochondrion. As an essential cofactor for mitochondrial α-ketoacid dehydrogenases, LA plays an important role in mitochondrial energy metabolism (Shay et al., 2009). Other than synthesis, it can be absorbed from diets, and accumulates quickly in different tissues. α-LA and its reduced form dihydrolipoic acid (DHLA) have been defined as an effective biological antioxidant, detoxification agent, diabetes medicine; and potential to improve age-related cardiovascular, cognitive, and neuromuscular diseases, and associated in modulating different inflammatory signaling pathways (Han et al., 1997; Anuradha et al., 1999; Liu et al., 2002; Suh et al., 2004; Smith et al., 2004).

Common food sources of LA are muscle meats, heart, kidney, and liver, and a small amount in fruits and vegetables also (Packer et al., 2001; Wollin and Jones, 2003). Takaishi et al. (2007) reported using a CACO-2 cell as transwell model for transepithelial transport that LA rapidly uptaken from dietary sources through the cell monolayer in a pH-dependent manner. *In vitro* studies acknowledged LA as a substrate for the Na^+-dependent multivitamin transporter, which may be responsible for its gastrointestinal uptake (Balamurugan et al., 2005; Prasad et al., 1998). It rapidly accumulates in the liver, heart, skeletal muscle, and other tissues. LA was reported to cross the BBB in few studies. Rats exposed to 25 mg/kg b.w. of LA was found to accumulate it in cerebral cortex within 60 min (Panigrahi et al., 1996), both young and old rats exposed to 100 mg/ kg b.w. of LA for 7‑14 days detected accumulation of LA in various brain areas (Arivazhagan et al., 2002).

LA stimulates diverse cellular mode of actions, from a potent antioxidant to a metal chelator and a modulator of cell signaling pathways. LA is one of the most effective naturally occurring antioxidants, because of its oxidized (α-LA) and reduced (DHLA) forms that generate a potent redox couple with reduction potential of −0.32 V (Shay et al., 2009). Both α-LA and DHLA can scavenge different type of reactive oxygen species, for example, hydroxyl radicals and hypochlorous acid; though both are unable to scavenge hydrogen peroxide; however, LA also scavenges singlet oxygen; (Shay et al., 2009). Moreover, DHLA

seems to regenerate other endogenous antioxidants (e.g. glutathione, vitamins C and vitamin E) (Bast and Haenen, 2003).

Furthermore, both α -LA and DHLA form chelate with a number of redox-active metals *in vitro* and *in vivo*. α-LA specially complexes with Cu^{2+}, Zn^{2+} and Pb^{2+}; however, it cannot bind to Fe^{3+}, whereas DHLA chelates with Cu^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+} and Fe^{3+} (Ou et al., 1995). DHLA impeded Cu(II)(histidine)2-mediated ascorbate oxidation strongly in a concentration-dependent manner, but not α-LA (Suh et al., 2005). On the other hand, only DHLA prohibited Cu (II)-mediated oxidation of LDL *in vitro* (Lodge et al., 1998). In the brain DHLA chelated with Fe^{3+} and Cu^{2+}, and improved the condition of Alzheimer's disease by depressing free radical damage (Bush et al., 2002).

Additionally, LA was reported to intensify eNOS activity, activate the transcription factor Nrf2 and akt-kinase and conformational changes in proteins by creating mixed disulfides, and suppress NF-kappa B (Shay et al., 2009) (Fig. 1.3).

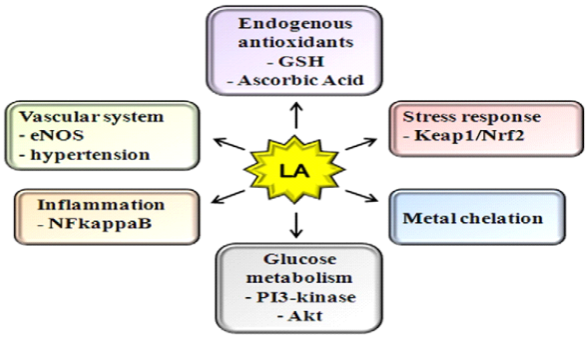

Figure 1.3: Proposed biological actions of lipoic acid (Shay et al., 2009).

1.6.3. BHT

Butylated hydroxytoluene (BHT) is a synthetic antioxidant (chemical name: 4-methyl-2,6-ditertiarybutylphenol) and the structural formula is shown in Fig. 1.4. The maximum permitted levels (MPLs) to use in foods is up to 100 mg/kg (expressed as fat) alone or in combination with other antioxidants such as gallates, tert-butylhydroquinone (TBHQ) and butylated hydroxyanisole (BHA) (EFSA, 2012). BHT has low acute toxicity. The acute oral LD50 of BHT was 1,700-1,970 mg/kg bw in rats, 2,100-3,200 mg/kg bw in rabbits, 10,700 mg/kg bw in guinea pigs, 940-2,100 mg/kg bw in cats, and 2,000 mg/kg bw in mice (Madhavi et al., 1996).

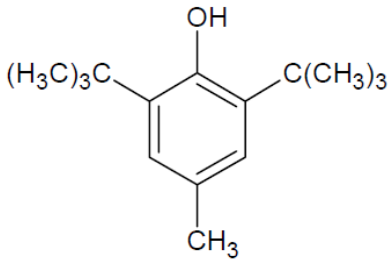

Figure 1.4: structural formula of BHT.

Absorption, distribution, metabolism and excretion of BHT have been studied in mice, rats, rabbits, chickens, monkeys and humans, though are not concluded concrete yet. BHT is absorbed promptly from the gastrointestinal tract and distributed. After absorption, it is generally distributed into the liver and body fat. BHT is metabolized in the liver to a variety of metabolites including sulfate and glucuronic acid conjugates (Babich, 1982). Excretion is majorly through urine and faces.

Numerous studies have been published about antioxidant activity of BHT and its use in food industries, with several toxicological studies. However, protective effect of BHT on toxicity due to toxic agents is very few. Al-Malki et al. (2010) reported antioxidant properties of BHT in carbon tetrachloride (CCl_4)-induced mice liver injury and BHT was more potential as an antioxidant than thymol. BHT-pretreated mice showed escalated antioxidant enzymes activities with simultaneous reduction in malondialdehyde (MDA) level and ALT as compared to CCl_4-treated rats. BHT-pretreatment reversed necrotic injury induced by CCl_4. Al-Malki et al. (2010) suggested that BHT-pretreatment ameliorates harmful effects of CCl_4 by augmenting the antioxidant activities.

Moreover, BHT has been used as a strong standard antioxidant in several toxicological studies. For instance, *in vivo* and *in vitro* studies were conducted using BHT against toxic ferric ion (Ko and Godin., 1990), Cd (Peters et al., 1995), Co (Lison et al., 1995), Cr (Pourahmad and O'Brien, 2001), mancozeb (Tsang and Trombetta, 2007), Cu (Hosseini et al., 2014), and Cu oxide (Assadian et al., 2017). BHT was also used as antioxidant standard in evaluating properties of melatonin (Gulcin et al., 2003).

Antioxidant activity of BHT in food processing has been studied extensively; however, its ameliorative role as an antioxidant in xenobiotic-induced toxicity remediation in biomolecular levels needs further investigation.

1.6.4. Zn

Zn is a trace element for nutritionally essential, vital to the structure and function of many macromolecules including enzymes modifying cellular processes and signaling pathways. It reulates immune response, and delivers antioxidant and anti-inflammatory property (Jarosz et al., 2017; Marreiro et al., 2017). In the human body, Zn is second most predominant trace element, after iron (Vasˇa´k and Hasler, 2000). In adults, the total amounts of Zn are 1.4‑2.3 g; however the quantity differs significantly in diverse tissues. For examples, the muscles and bones contain 85% of total Zn, where skin and liver contain 11%, and rest of 4% in other tissues of the body (Calesnick and Dinan, 1988). Major food sources of Zn are sea food, beef, lamb, eggs, whole grains, nuts and yogurt (Barceloux, 1999). According to the Institute of Medicine, Food and Nutrition Board (2001), the recommended daily allowance (RDA) of Zn for adults is 8-11 mg.

Acrodermatitis enteropathica is a disorder caused by incapability to absorb adequate Zn from diet. Zn deficiency was reported to cause growth retardation, poor appetite, impaired immunity, mental lethargy, cognitive impairment, symptoms of depression, Alzheimer disease, enhanced oxidative stress and increased generation of inflammatory cytokines (Jarosz et al., 2017).

Antioxidant defense activity of Zn has been studied extensively. Zn contributes in GPX regulation, MT formation. Additionally, Zn competes with Fe and Cu at the cell membrane, constrains the NADPH-oxidase, and decreases chronic inflammation and hyperglycemia (Cruz and Oliveira, 2015; Lima and Sampaio, 2011).

Zn is a structural component of superoxide dismutase (SOD), which promotes the conversion of toxic ROS from a highly reactive to a less harmful form (Cruz and Soares, 2011). Also, Zn deficiency was found to encourage synthesis of SOD and results in protein synthesis inhibition and Zn transporter Zip-14 initiation (Homma and Fujisawa, 2013).

Alternative Zn-mechanism is modulation of glutamate-cysteine ligase, the rate-limiting enzyme of GSH synthesis. Hence, Zn neutralizes free radicals directly by GSH or indirectly as a GSH peroxidase cofactor (Eide, 2011). Ha and Chen (2006) reported that Zn-administration upregulates the mRNA levels of glutamate-cysteine ligase through Nrf2 pathway in ARPE-19 cells. Foster and Samman (2010) also reported that Zn regulates the total concentration of GSH in the cells.

Zn can induce MT, which functions as a preferred sacrificial site for oxidant attacks, preserving the cellular components. MT is capable of binding heavy metal ions, such as Zn,

Cu, Cd and Hg effectively (Coyle and Philcox, 2002; Günther and Davis, 2012; Günther and Lindert, 2012). Zn is released from its complex with MT in oxidative stress situations and is redistributed in the cells to exert antioxidant actions (Maret and Krezel, 2007; Özcelik and Nazıroglu, 2012). Zn-supplementation induced increased MT and its favor in antioxidant and anti-inflammatory effects in the liver of rats were observed (Liang and Zhang, 2015).

Responsive metal transcription factor 1 (MTF-1) is Zn-dependent transcription factor which stimulates MT and zinc transporter-1 (ZnT-1) gene expressions in high Zn concentrations. MTF-1 initiates Selenoprotein 1 gene expression, which encodes GSH-binding protein that scavenges free radicals (Bonaventura and Benedetti, 2015). Additionally, MTF-1 regulates the immune response by regulating the gene expressions of inflammatory cytokines (Grzywacz and Gdula-Argasi´nska, 2015). Moreover, Zn-supplementation was also reported to employ protection against vascular damage by the regulation of Nrf2 (a transcription factor) which is important factor for antioxidant enzymes and MT induction (Biagiotti and Menotta, 2016; Jenner and Ren, 2007; Miao and Wang, 2013).

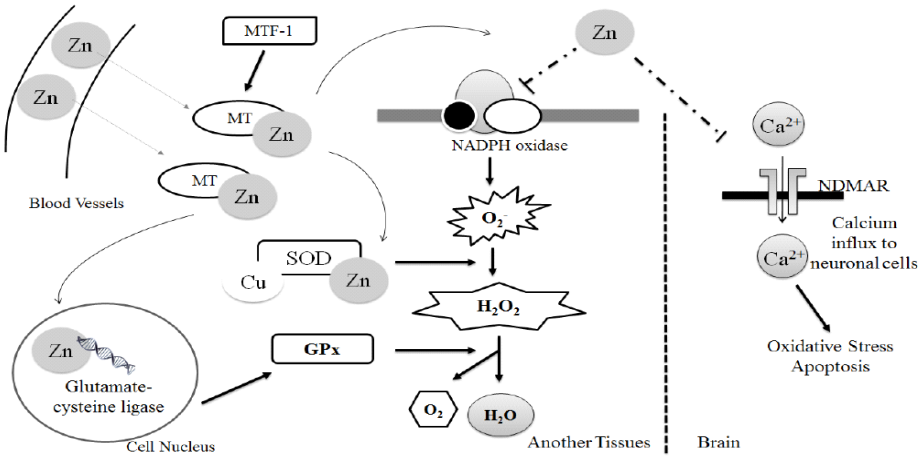

Figure 1.5: Zinc participation in antioxidant mechanisms. GPx: Glutathione peroxidase; MT: Metallothionein; MTF-1: Metal-responsive transcription factor 1; NADPH: nicotinamide adenine dinucleotide phosphate; NMDAR: N-methyl-D-aspartate receptor; SOD: superoxide dismutase enzyme; Zn: Zinc (Marreiro et al., 2017).

As Zn is an inhibitor of N-methyl-D-aspartate (NMDA) receptors, in Zn deficiency NMDA receptors are activated and intracellular Ca concentration increases, which in turn contributes in inflammatory cytokines and free radicals production, resulting in increases of oxidative stress (Weglicki and Chmielinska, 2011). Furthermore, NADPH oxidase and nitric oxide

synthase are activated in Zn deficiency, consequently results in increases of ROS and nitrogen species (Oteiza, 2012) (Fig. 1.5).

1.7. Research motivation

The motivation of my current research is basically"Food components could be one of the therapeutics for the toxic metals induced diseases". In this segment some important particulars behind the research motivation are explained. Bangladesh has been exposed with a threat situation of heavy metals pollution in environment. UNEP (ESDO & UNEP, 2015) reported an alarming situation of Hg pollution in Bangladesh as resulted from the uses of Hg and Hg-containing products. According to the report Hg released into the environment from industries, such as chlorine (Cl_2) producing industries (4.49 MT/year), cement industries (0.14 MT/year), aluminum production companies (total emission of 0.011 MT into air) and steel production process (0.16 MT into air). In addition, based on their survey, a person inhales Hg during amalgam dental fillings on an average 3-17 µg/day and 1,095-6,205 mg/year. It meant that 1.09-6.22 MT/year Hg vapor is released from the dental sector in Bangladesh (ESDO & UNEP, 2015). Lack of proper knowledge about disposal causes generation of 1.12 MT/year Hg waste, and releases into environment via waste deposition, land filling and waste water treatment. In addition, annually 0.170 MT of Hg emissions from cremation has been added (ESDO 2012a). Moreover, 12 cosmetic products in Bangladesh were reported to contain 3,000 to 5,000 ppm of Hg by ESDO (2012b). Significant Hg concentrations in sediment, poultry feeds and fishes were also reported in Bangladesh (Siddiquee et al., 2012; Islam et al., 2007; Holsbeek et al., 1997). Not only in Bangladesh, as Hg is a universal environmental pollutant, severe Hg pollution in the environment (atmosphere, lake water, lake sediments and catchment soils, soil feeding termites) of anthropogenically-disturbed sites (Karunasagar et al., 2006; Wang et al., 2014; Zhang et al., 2019; Karunasagar et al., 2006; Yang et al., 2016; Cheng et al., 2020; Diouf et al., 2019) and dreadful contaminations in food sources (fresh and marine fish, rice, clam) (Karunasagar et al., 2006; Zupo et al., 2019; Yoshino et al., 2020; Xu et al., 2020; Sıkdokur et al., 2020) across the world has been reported. Hence, Hg exposure and potential associated risk factor to human health are a global concern.

Moreover, another toxic environmental toxicant Cd has been identified in food items of Bangladesh, for instance fish (0.1 mg/kg), pasteurized cow's milk (0.053 mg/kg) and dairy milk (0.024 mg/kg), honey (0.024 mg/kg), frozen shrimp (0.043 mg/kg) and fish (0.13 mg/kg), chicken egg (0.3 mg/kg), duck (0.34 mg/kg), raw rice (0.03 mg/kg), cooked rice

(0.047 mg/ kg), bitter gourd (0.021 mg/kg), and in eggplant (0.027 mg/kg) (Khan et al., 2010; Dar et al., 2017; Kippler et al., 2012a; Shaheen et al., 2016; Kippler et al., 2012b; Ullah et al., 2017), indicating an alarming health condition.

Up to now there is no perfectly suitable therapeutic to detoxify the metals in biological system. This deleterious metal pollutions and potential fatal health conditions motivated me to investigate the basic mechanisms of toxic heavy metals in biological system and to explore for potential cure using food components to diminish the toxic effects of metals. Therefore, it was hypothesized that "Food components (Se, DHLA, BHT and Zn) have regulatory effects on toxic heavy metals mediated toxicity in cellular system" because these compounds have the ability to improve the oxidative state in the body.

1.8. Aim and objectives

The foremost aim of my research is to explicate the fundamental mechanisms of heavy metals mediated cytotoxicity and their remediation using food components by the usage of some bio-molecular toxicology assessment. To accomplish this goal, particular objectives in each chapter were specified as follows:

- To prepare an overview of existing literatures to evaluate the current progresses and to stipulate required exploration.
- To investigate the cytotoxicity of food components (Se, DHLA, Zn and BHT) using mammalian cells.
- To evaluate the potential protective effects of food components (Se, DHLA, Zn and BHT) on heavy metals (Cd and Hg)-induced toxicity in cellular level.
- To clarify the molecular mechanism/s responsible for the cyto-protection of food components (Se, DHLA, Zn and BHT) against metal-induced toxicity.

1.9. Outline of the thesis

This thesis comprised of seven sequential chapters including the first chapter on general introduction. In chapter 1, a comprehensive background exploration about heavy metal (Cd and Hg) pollutions was described focusing on the sources of exposure, tolerable limits, toxicokinetics, and their effects on human and other biological system. In addition, a brief discussion was conducted aiming on biological functions and antioxidant abilities of Se, Zn, DHLA and BHT in biological systems. Moreover, a detailed overview and purposes of the present study were elucidated on protective roles of the diet components against toxic metals.

After that, chapter 2 will discuss the protective effects of Se against Cd-induced cytotoxicity in PC12 cells and its bio-molecular mechanisms. In addition, the cytotoxic effects of Se will be also presented with its molecular mechanisms. Hence, Se-induced autophagy cell death in PC12 cells was found for the first time. The regulatory effects of DHLA against inorganic Hg-mediated cytotoxicity and intrinsic apoptosis in PC12 cells will be depicted in Chapter 3. Then in the chapter 4, it was discussed that Se protects inorganic Hg-induced cytotoxicity in PC12 cells. Here, the mechanisms of protection of Hg toxicity by Se were explained as reducing oxidative stress and intrinsic apoptosis. In chapter 5, the protective effects of BHT on inorganic Hg-induced cytotoxicity and intrinsic apoptosis in PC12 cells with its underlying molecular mechanisms were discussed. In chapter 6, the effects of Zn-pretreatment on inorganic Hg-induced cytotoxicity and their bio-molecular mechanisms were discussed using PC12 cells. Finally, in chapter 7 an overall discussion and conclusion will be given. This chapter will summarize all the outcomes focusing individual molecular mechanisms in distinct toxic situations.

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Chapter 2: Inhibitory effects of selenium on cadmium-induced cytotoxicity in PC12 cells via regulating oxidative stress and apoptosis

Abstract

Purpose of this study is to investigate mechanism/s of cyto-protection by selenium (Na_2SeO_3; Se^{4+}) against cadmium ($CdCl_2$; Cd^{2+})-induced cytotoxicity using PC12 cells. In addition, Se (5, 10, 20 and 40 μM) and Cd (2.5, 5 and 10 μM)-induced cytotoxicity is determined. Cytotoxicity assays and western blot analyses confirmed that Se (≥10 μM) promotes autophagic cell death via inhibition of mTOR activation and p62 accumulation due to increase of cellular oxidative stress. On the other hand, co-presence of non-toxic Se (5 μM) and toxic Cd (5 μM) showed to increase cell viability, glutathione and glutathione peroxidase 1 (GPx1) levels, and to decrease DNA fragmentation and lactate dehydrogenase (LDH) activity compared to Cd-treated (5 μM) cells alone. Furthermore, western blot analyses of cytochrome c and ERK1 indicated that Cd-induced apoptotic cell death in PC12 cells. However, the co-exposure of Se with Cd significantly decreases the release of cytochrome c into cytosol from mitochondria, and up-regulates ERK1 protein to inhibit Cd-induced apoptosis. In conclusion, Se (≥10 μM) possess cytotoxicity in PC12 cells; however, co-presence of Se (5 μM) with Cd (5 μM) protects against Cd-induced apoptosis in PC12 cells due to inhibition of Cd-induced oxidative stress and subsequently suppression of mitochondrial apoptosis pathway.

2.1. Introduction

Selenium (Se), one of trace elements, is an essential nutrient in human biology, and has important structural and enzymatic roles as a component of selenoproteins. It is well-known as antioxidant and anti-inflammatory agents, and it can protect from cardiovascular diseases. Se is required for the proper functioning of immune system, metabolism of thyroid hormone, regulation of virulence, disease progression of some viral infections (such as HIV), successful reproduction (such as sperm motility and reduced risk of miscarriage), controlling mood status and reducing cancer risk (Rayman, 2000, 2012). An adequate Se intake has been estimated as 55 μg/day, and deficiency of Se is associated with several diseases such as immune impairment and Keshan disease (Rayman, 2000; 2012; USDA, 2012). In addition, low dose of Se intake as a supplement is beneficial for cancer prevention, and it also can encourage many other functions in an organism by reducing inflammations and oxidative

damage (Hatfield et al., 2011). Consumption of Se greater than the recommended daily allowance (RDA), can be shown cytotoxic effects on cells. However, it provides protection against certain types of cancers (Fordyce, 2013; Hatfield et al., 2011). Se is involved in function of several proteins and enzymes, and in the form of seleno-cysteine, the 21st amino acid, is recognized as selenoproteins. Twenty-five classes of selenoproteins have been identified as glutathione peroxidases (GPxs), selenoproteins N, S, P, W and R, thioredoxins and so on. Several of them have been reported to deliver antioxidant activity; however, at low Se-supply, the synthesis of GPxs is prioritized over that of others (Reeves and Hoffmann, 2009). All members of GPx group catalyze the reduction of hydrogen peroxide and/or phospholipid peroxides using glutathione (GSH), and provide agreat scale of antioxidant protection (Reeves and Hoffmann, 2009).

Recently, although Se has been a concern of interest for both of its antioxidant and anticancer properties, the studies achieved in this regard are still not sufficient. Though Se is beneficial for human body, excess Se intake has been reported to be toxic for the organisms. It is scientifically proved that high dose of Se induces oxidative damage and cell death (Li et al., 2003; Zuo et al., 2004; Guan et al., 2009). That is why searching the threshold concentration, above which it has cytotoxic effects on organisms, is crucial. Se induced cytotoxicity in different cell lines is mostly through apoptosis induction (Luo et al., 2012; Abdulah et al., 2011). However, the mechanism behind the toxic effects of Se is still inconclusive.

Cadmium (Cd) is a toxic environmental pollutant which received great concern worldwide for its poisonous effects on human health. It was classified by International Agency for Research on Cancer (IARC) as Group I carcinogen to humans. Cd accumulation contributes to carcinogenesis, immune depression and neurodegenerative diseases (Chen et al., 2008a, 2008b). Exposure of Cd causes pulmonary edema, respiratory tract irritation, renal dysfunction, anemia, osteoporosis, cancer and neurological disorder (Chen et al., 2008b; Lau et al., 2006). Olfactory dysfunction and neurobehavioral defects were induced in Cd exposed workers (Lau et al., 2006). Children exposed to Cd were observed to have neurological disorders including learning disability and hyperactivity (Chen et al., 2008b). Parkinson's disease and Alzheimer's disease are believed to be associated with Cd-induced oxidative stress (Chen et al., 2008a, 2008b). Recently, studies have shown that Cd delivers toxicity in different cell lines including PC12 cells via generation of reactive oxygen species (ROS) and oxidative damage induction (Jiang et al., 2014; Rahman et al., 2017).

In this study, PC12 cell line has chosen to use as a model cell line for toxicity assessment (Magalingam et al., 2014; Rahman et al., 2017, 2018). Here, PC12 cell line was used for the

first time to assess Se-toxicity and to examine the potential cytoprotective role of Se against Cd-toxicity. Protective effects of antioxidant trace metals upon simultaneous exposure with another toxic metal have been reported recently using different cell cultures. For instance, zinc has been reported to reduce Cd-induced cytotoxicity in PC12 cells upon co-exposure by reducing oxidative stress and inhibiting apoptosis (Rahman et al., 2017); ameliorative effects of selenium upon co-exposure with arsenic have been reported in PC12 cells by positively regulating autophagy/apoptosis (Rahman et al., 2018), while a dose dependent effects of selenite and arsenite co-exposure was reported in NB4 cells to modulate cellular apoptosis (Wang et al., 2015). In addition, protection of Se against Cd induced toxicity has been studied in-vivo (El-Sharaky et al., 2007; Li et al., 2010) and in-vitro (Liu et al., 2007; Zhou et al., 2009; Chen et al., 2012). However, the exact biomolecular mechanism/s of cytoprotective effects of Se against Cd-induced toxicity remains inconclusive. Moreover, the antioxidant/cytoprotective effects of Se on Cd-induced toxicity in PC12 cells are not reported. Thus, in this study, it was hypothesized that Se have alleviative effects against Cd-toxicity in PC12 cells. In addition, Se-induced cytotoxicity in PC12 cells was investigated to clarify the mechanisms for the first time in light of autophagy or type II cell death. Therefore, to investigate the above mentioned hypothesis certain cytotoxicity assessment techniques were employed after co-/ exposure of Se and/or Cd in PC12 cells for a specified period of incubation such as cell viability analysis, DNA fragmentation analysis, lactate dehydrogenase (LDH) activity analysis, GSH contents analysis and protein expression analysis by western blotting.

2.2. Materials and methods

2.2.1. Reagents and antibodies

PC12 cells were purchased from the American Type Culture Collection (USA and Canada). Fetal bovine serum (FBS) was bought from Biosera (Kansas City, MO, USA). Dulbecco's modified Eagle's medium (DMEM), ribonuclease A (RNase), ethidium bromide and peroxidase conjugated avidin were purchased from Sigma (St. Louis, MO, USA). Polyclonal antibodies against β-actin (GTX 109639, GeneTEX), GPX-1 (AB22604, Abcam), caspase-3 (CS3-PA001231), active caspase-3 (AB2302, Abcam), mTOR (A301-144A-T, Cosmo Bio Co., LTD), phosphorylated mTOR (SC-293133, Cosmo Bio Co., LTD), p62 (8025, Cell Signaling Technology), ubiquitin (#3933S, Cell Signaling Technology), ERK1 (610031, BD Transduction laboratories) were purchased. Anti-cytochrome c monoclonal antibody was

purchased from BD Biosciences Pharmingen (San Jose, CA, USA). Trypan blue solution (0.4%) was purchased from Bio-Rad (Hercules, CA, USA). ECL western blotting detection reagent was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Proteinase K was purchased from Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade.

2.2.2. Cell culture

PC12 cells were maintained in DMEM supplemented with 10% FBS in a humidified incubator with 5% CO^2 at 37 °C. The cells were grown in 25-cm^2 flasks for 48 h. After that, the medium was replaced with serum/serum-free DMEM with or without various concentrations of Na_2SeO_3 (Se^{4+}) as a source of Se, and the cells were incubated for 48 h at 37 °C. The desired concentration for treatment was selected by treating PC12 cells with different concentrations of Na_2SeO_3, and then the final combination was selected. The selected concentration for Na_2SeO_3 was 0, 5, 10, 20 and 40 μM.

Again, after 48 h the medium was changed with DMEM containing 10% FBS and treated with or without various concentrations of $CdCl_2$ (Cd^{2+}) and Na_2SeO_3 (Se^{4+}), or with a mixture of both. The desired concentration for treatment was carefully chosen by exposing PC12 cells to Cd (0, 2.5, 5 and10 μM) and Se (0, 5, 10, 20 and 40 μM) separately, and then the final combination was selected. The chosen concentration for both metals was 5 μM. The cells were incubated for 48 h.

2.2.3. Cell viability

Cell viability was analyzed using trypan blue exclusion assay. PC12 cells were cultured and preincubated for 48 h in DMEM supplemented with 10% FBS. Then, the cells were treated with and without Na_2SeO_3 and $CdCl_2$. After 48 h of incubation total cells and trypan blue-staining cells were calculated using a Bio-Rad automated cell counter (Hercules, CA, USA). Each experiment was done at least six times to confirm biological reproducibility.

2.2.4. Lactate dehydrogenase (LDH) activity assay

Cell-wall integrity was analyzed by LDH activity assay of the medium treated with Na_2SeO_3 using a non-radioactive cytotoxicity assay kit (Promega; Fitchburg, WI, USA) as described by Rahman et al. (2017). PC12 cells were cultured in the medium with/without Na_2SeO_3 (5, 10, 20, and 40 μM) for 48 h. After the incubation, 50 μL of the medium was taken to a 1.5 mL tube, and then 50 μL of a substrate mixture containing tetrazolium salts was added to the tube. The mixture was kept at room temperature (around 25 °C) for 30 min. After 30 min 50

µL of stop solution was added. The amounts of formazan dye formed were assessed by determining the absorbance at 490 nm with a iMark™ immunoplate Reader (BioRad; Hercules, CA, USA). LDH activity was expressed as LDH activity/1×10^6 cells. Similar process was done for LDH assay of the PC12 cells that were cultured with/without Na2SeO3, $CdCl_2$ and combination of Na_2SeO_3 and $CdCl_2$. These experiments were performed three times for confirming reproducibility.

2.2.5. Isolation of genomic DNA from PC12 cells

PC12 cells were exposed to Na_2SeO_3 (0, 5, 10, 20, and 40 µM), $CdCl_2$ (5 µM) and combination of Na_2SeO_3 and $CdCl_2$ (5 µM + 5 µM); and after 48 h of incubation the cells were harvested. Cells were washed with 1 × PBS and the genomic DNA was isolated from the cells using high pure PCR template preparation kit (Roche Diagnostics; Mannheim, Germany) as described by Kawakami et al. (2008). After washing the cells with 1×PBS, 200 µL PBS, 200 µL binding buffer (10mM Tris-HCl, pH 7.4, containing 6 M guanidine-HCl, 10 mM urea and 20% Triton X-100) and 40 µL proteinase K (20 µg/ml) were added and incubated at 70 °C for 10 min. After 10 min 100 µL isopropanol was added to the mixture and the solution was transferred into a high pure filter tube combined with a collection tube. The solutions were centrifuged at 12,000 rpm for 1 min. After the centrifugation, 500 µL inhibitor removal buffer (5 M guanidine-HCl and 20 mM Tris-HCl) was added to the filter tube attached with a new collection tube, and the mixture was centrifuged at 12,000 rpm for 1 min. The obtained precipitate was washed twice with 500 µL washing buffer (2 mM Tris-HCl, pH 7.5, containing 20 mM NaCl). After the centrifugation, 200 µL elution buffer which was pre-warmed at 70 °C, was added to the filter tube attached with a 1.5 mL microcentrifuge tube (Eppendorf, Germany) and centrifuged at 12,000 rpm for 1 min to obtain the solution containing DNA. After adding 2 µL of 500 µg/mL RNase, the DNA solution was incubated for 15 min at 37 °C. After the incubation, 500 µL ethanol and 20 µL of 3 M NaOAc buffer, pH 4.5, were added for ethanol precipitation, and the solution allowed to stand overnight at −20 °C. After suspending precipitated DNA in 1× Tris/Borate/EDTA (TBE), DNA concentration was measured with a Personal Spectrum Monitor (Gene Quant Pro, GE, USA).

2.2.6. Agarose gel electrophoresis of genomic DNA

The amount of DNA fragmentation was observed using agarose gel electrophoresis. The adequate amounts of DNA in the cells treated with Na_2SeO_3 (0, 5, 10, 20 and 40 µM), $CdCl_2$ (5 µM) and combination of Na_2SeO_3 and $CdCl_2$ (5 µM + 5 µM) for 48 h were subjected to electrophoresis on a 1.5% agarose gel. Electrophoresis was carried out for 30 min at 100 V by

using a submarine-type electrophoresis system (Mupid-ex, Advance, Tokyo, Japan). To visualize the DNA fragments, the gel were soaked in ethidium bromide solution for 10 min. Images of the agarose gel were taken by UV illumination using a ChemiDoc XRS (Bio-Rad; Hercules, CA, USA). For assessing DNA fragmentation, the fluorescence intensity of DNA in the gel was analyzed by a software named Quantity one. This experiment was conducted 3 times at least.

2.2.7. Measurement of intracellular free sulfhydryl (SH) levels

Intracellular free SH levels were investigated according to the method described by Kihara et al. (2014) and Rahman et al. (2017). Cells were pre-incubated for 48 h and exposed to Na_2SeO_3 (0, 5, 10, 20, and 40 μM), $CdCl_2$ (5 μM) and combination of Na_2SeO_3 and $CdCl_2$ (5 μM + 5 μM). After 48 h the cells were harvested, washed with 1 × PBS, added to 150 μL of a lysis buffer, and then incubated for 10 min at 4 °C. To break the cell membranes two freeze-thawing and sonication cycles were performed, and the resultant solution was centrifuged at 1500 rpm for 10 min. After that the supernatant collected and the total protein content of the supernatants were measured by spectrophotometric method using protein assay dye reagent (Bio-Rad; Hercules, CA, USA). Intracellular free SH levels in the cell lysate were determined using 2.5 μM of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm wavelength with a iMark™ immunoplate Reader (BioRad; Hercules, CA, USA). The concentration of free SH in PC12 cells was assessed using a molecular coefficient factor of 13,600 per cell number (1×10^6). The experiments were done three times to ensure mechanical reproducibility.

2.2.8. Western blot analysis for determination of protein expression

PC12 cells cultured in DMEM containing 10% FBS with/without Na_2SeO_3 (5, 10, 20, and 40 μM), $CdCl_2$ (5 μM) and combination of Na_2SeO_3 and $CdCl_2$ (5 μM + 5 μM) were harvested. After addition of 5 mL of ice-cold PBS, the mixture was centrifuged at 1500 rpm for 5 min and the supernatants were removed. The cells were resuspended in 150 μL of lysis buffer (2 mM HEPES, 100 mM NaCl, 10 mM EGTA, 1 mM PMSF, 1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 5 mM β-glycerophosphoric acid disodium salt, 50 mM NaF, 1 mM $MgCl_2$, 2 mM DTT and 1% TritonX-100) and kept in 4 °C for 10 min. Then two cycles of sonication followed by centrifugation at 1500 rpm for 10 min, were done to collect the protein fraction in the lysis buffer and the lysate ware separated from the precipitate. After that, the protein concentration of the lysate was determined spectrophotometrically by using protein assay dye reagent (Bio-Rad, Hercules, CA, USA). Then, the equal amount (20 μg) of protein was separated by polyacrylamide gel electrophoresis (12.5–15%), and the electrophoresed

proteins were shifted to a nitrocellulose membrane with a semidry blotting system, type-AE6678 (ATTO, Tokyo, Japan). The membranes were incubated overnight at 4 °C in 5% skimmed milk as a blocking agent. Then the membranes were washed three times and incubated for 60 min at 37 °C with the primary antibodies, again washed three times, and then incubated with the secondary antibody for 60 min. After washing for five times (each time 3 min), the protein bands were visualized using enhanced chemiluminescence. The images of the detected bands were analyzed using a ChemiDoc XRS (Bio-Rad, USA). Each experiment was performed at least in triplicate to ensure reproducibility.

2.2.9. Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Tukey-Kramer's test.

2.3. Results

2.3.1. Cell viability

To investigate Se effects of cell toxicity on PC12 cells, cells were exposed to 0, 5, 10, 20 and 40 μM of Se. Fig. 2.1A shows the cell viability of cultured PC12 cells after 48 h of incubation with different concentrations of Se using trypan blue staining. The cell viability of PC12 cells was not reduced by treatment with 5 μM Se for 48 h; however, it was significantly decreased with high concentrations of Se (20 and 40 μM) ($P < 0.05$) as compared with the control group (no treatment). The cell viability was reduced to 87% when the PC12 cells were treated with 10 μM of Se. In addition, 20 μM and 40 μM of Se caused decreasing cell viability to 50% and 28%, respectively. The cell viability study indicated that higher concentrations of Se (20 and 40 μM) were toxic for PC12 cells.

Similarly, the cell viability of the combined treatment was analyzed using trypan blue straining assay. As shown in Fig. 2.1B, cell viability of PC12 cells treated with different concentration of Cd (0, 2.5, 5 and 10 μM) for 48 h. The cell viability of PC12 cells treated with greater than 2.5 μM of Cd showed significant decrease compared to the cells of no treatment (Fig. 2.1B) which indicated that Cd induced toxicity in PC12 cells. Fig. 2.1C showed cell viability of PC12 cells treated with Cd (5 μM), Se (5 μM) or both metals (5 μM + 5 μM) for 48 h. It was shown in Fig. 2.1C that the cell viability was significantly decreased ($p < 0.05$) compared to that of the cells without treatment of both metal ions, when the cells were treated with 5 μM of Cd; however, cell viability of the cells treated with only 5 μM of Se did not show any significant difference compared to that of the control cells. The viability

of PC12 cells exposed concurrently to both 5 µM of Cd and Se showed significant increase ($p < 0.05$) as compared with that of the cells treated with only 5 µM of Cd. These results suggest that Se can protect PC12 cells from Cd induced toxicity.

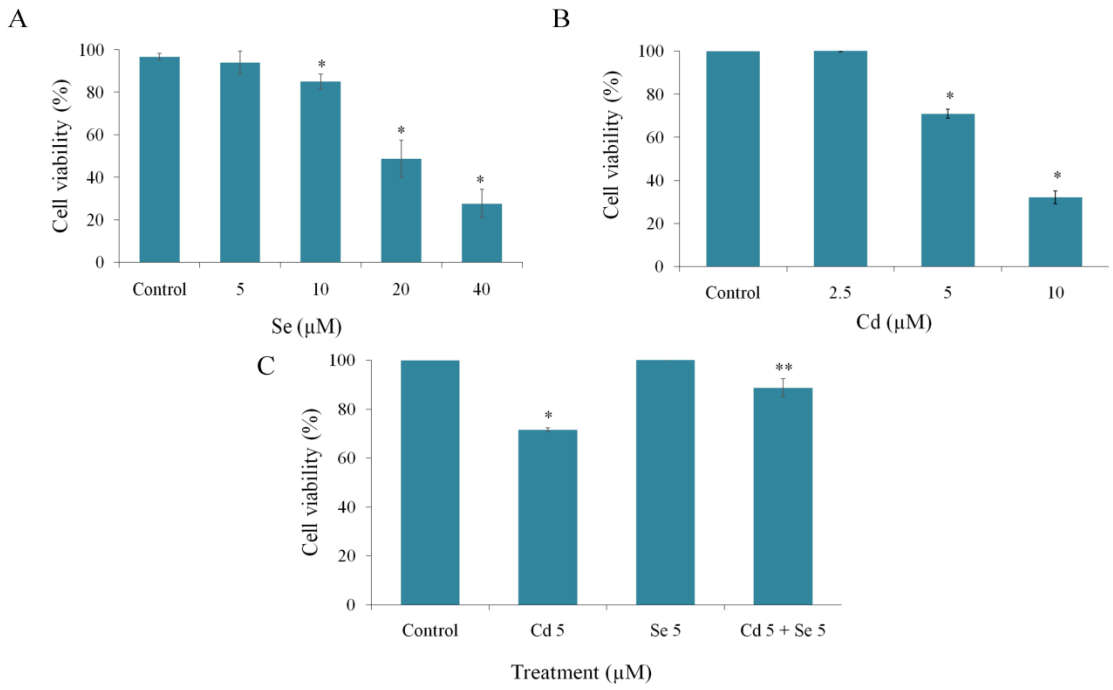

Figure 2.1: Cell viability of PC12 cells cultured in FBS containing medium with 0-40 µM of Se (A), 0-10 µM of Cd (B) and with/without Se (5 µM), Cd (5 µM) or both metals (5 µM + 5 µM) (C). Error bars indicate mean ± standard error (n=3). Statistical significance was evaluated by t-test: two sample assuming unequal variance, where * denotes $p < 0.05$, significantly difference from control group and ** denotes $p < 0.05$, significantly difference from only Cd-treated group.

2.3.2. Loss of cell-wall integrity

To examine effects of Se treatment on cell-wall integrity in PC12 cells, trypan blue staining of the cells and LDH activity assay of the culture medium were performed. The images of trypan blue staining showed in Fig. 2.2A. Dead cells indicating red color increased significantly when treated with high concentrations of Se (20 and 40 µM) compared to those in the control group (Fig. 2.2A). Fig. 2B shows the LDH activity in the culture media of the cells treated with 0, 5, 10, 20 and 40 µM of Se for 48 h. From the results, the levels of LDH activity in each treated cells were in good agreement with the results of trypan blue staining. It was showed that the treatment with high concentrations of Se (20 and 40 µM) in the cells had significant difference ($P < 0.05$) as compared with control group (no treatment) (Fig. 2.2B). It increased almost three times than that in control cells when the cells were treated

with 40 µM of Se. These results suggested that high concentrations of Se (20 and 40 µM) have cytotoxic effect on PC12 cells, and these toxicities may cause cell-wall damage. These results were in good agreement with the cell viability results (Fig. 2.1A).

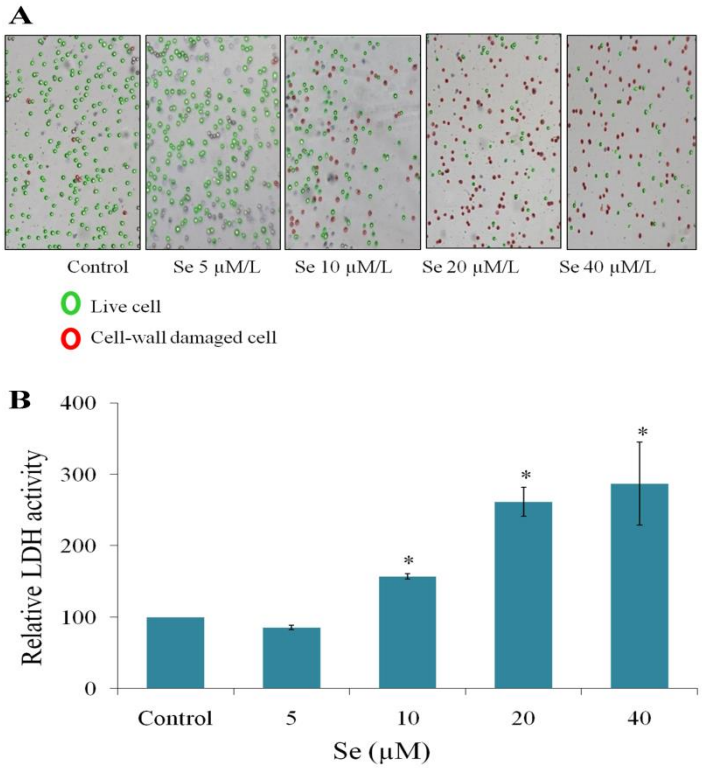

Figure 2.2: (A) Detection of cell wall damage in the cultured cells using trypan blue staining when cells were treated with Se (0-40 µM) for 48 h. (B) LDH levels in the cultured medium for PC12 cells treated with 0–40 µM of Se for 48 h. Each column is shown as mean ±SEM, n=3. Statistical significance was evaluated by t-test: two sample assuming unequal variance, where * denotes $p < 0.05$, significantly different from control group.

LDH activity in culture media for the PC12 cells treated with/ without Cd (5 µM), Se (5 µM) or both metals (5 µM + 5 µM) was investigated (Fig. 2.3A). LDH levels in the culture medium for the cells treated with Cd (5 µM) were significantly increased ($p < 0.05$) compared to that of the cells without treatment of both metal ions. It was suggested that Cd (5 µM) gives toxic effects on PC12 cells and the cell wall damage. On the other hand, when cells were exposed concurrently to both 5 µM of Cd and Se, LDH levels were reduced to the same level of control, because there is no significant difference between control and both metals (5 µM + 5 µM). These findings suggest that Se can reduce the toxic effects and cell wall damage induced by Cd. To confirm the protective effects of Se on Cd induced cell wall damage, PC12 cells were strained with trypan blue. Fig. 2.3B shows the images of cell wall

damages after incubation of the cells for 48 h with Cd (5 µM), Se (5 µM) or both metals (5 µM + 5 µM). The cell wall damaged cell was obviously decreased when cells were exposed to both Cd (5 µM) and Se (5 µM) together compared to those in the cells exposed to only Cd (5 µM). These results showed the same tendency against from trypan blue assay (Fig. 2.1C) and LDH activity assay (Fig. 2.3A).

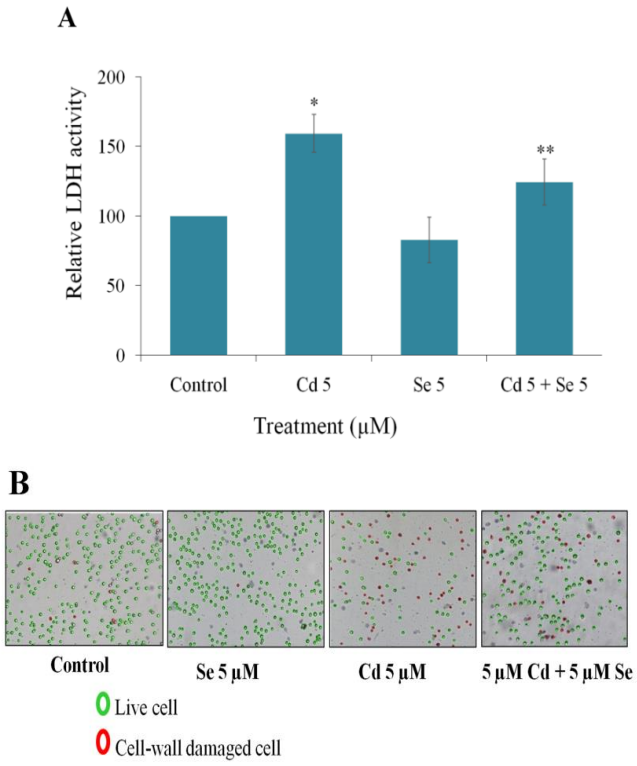

Figure 2.3: (A) LDH levels in the cultured medium for the PC12 cells treated with/without Cd (5 µM), Se (5 µM) or both metals (5 µM + 5 µM) for 48 h. Error bars are mean ± SEM (n=3). Statistical significance was evaluated by t-test: two sample assuming unequal variance, where * denotes $p < 0.05$, significantly difference from control group and ** denotes $p < 0.05$, significantly difference from only Cd-treated group. (B) Detection of cell wall damage in the cultured cells using trypan blue staining when cells were treated with the same condition as (A).

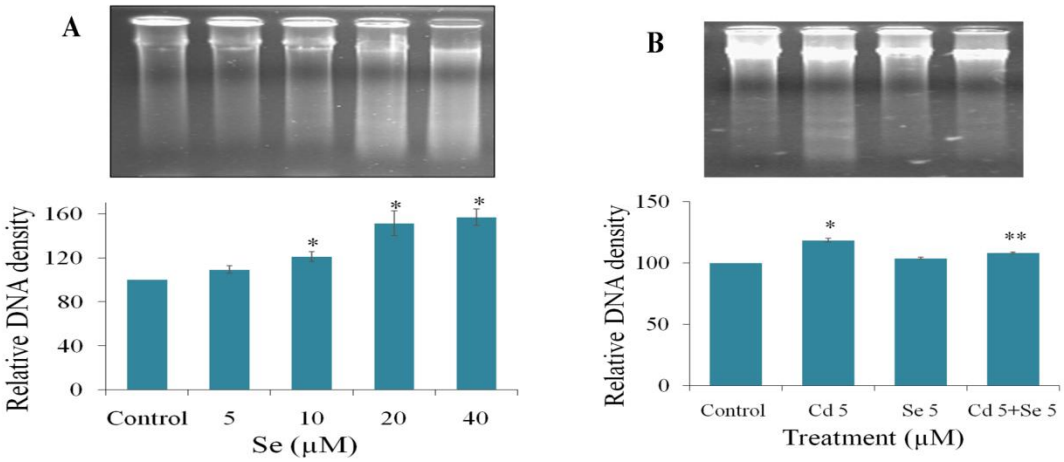

Figure 2.4: Agarose gel electrophoresis of DNA isolated from PC12 cells (A) treated with 0–40 µM of Se,(B) treated with/without Cd (5 µM), Se (5 µM) or both metals (5 µM + 5 µM) for 48 h.Intensity of band obtained from DNA in the cells treated was shown below the electrophoresis band.Error bars are mean ± SEM (n=3).Statistical significance was evaluated by t-test: two sample assuming unequal variance, where * denotes $p < 0.05$, significantly difference from control group and ** denotes $p < 0.05$, significantly difference from only Cd-treated group.

2.3.3. DNA fragmentation

To investigate whether the extracted DNA from PC12 cells treated with Se was damaged, DNA fragmentation was observed using agarose gel electrophoresis. The genomic DNA was extracted from the PC12 cells after the treatment with 0–40 µM of Se, and was electrophoresed using 1.5% agarose gel as shown in Fig. 2.4A. DNA ladder pattern which is an indication of apoptosis, was not observed in the profiles of DNA electrophoresis, although DNA was degraded depending on Se concentrations in the medium. These results suggested that the cell death occurred among the cells treated with Se, was not caused by apoptosis process. A significant increase ($P < 0.05$) of DNA degradation was observed while the cells were treated with high concentrations of Se (20 and 40 µM) for 48 h. In addition, the DNA degradation was increased significantly with increasing Se concentrations in the medium. Degree of DNA degradation is well coincided with cell viability of the cells treated with several concentration of Se (Fig. 2.1A). To observe the protective effects of Se on Cd induced DNA damage and/or apoptosis, genomic DNA was extracted from treated PC12 cells, and was electrophoresed. As shown in Fig. 2.4B, profiles of agarose gel electrophoresis of DNA isolated from PC12 cells treated with Cd (5 µM), Se (5 µM) or both metals (5 µM + 5 µM) for 48 h. The ladder formation which is a marker for apoptosis was observed when PC12

cells were exposed to Cd (5 µM); however, the ladder level was decreased to the same level of control when PC12 cells exposed concurrently to both 5 µM of Cd and Se. These results indicated that apoptosis induced by 5 µM of Cd was inhibited partially due to treatment of Se. These observations support the results from Fig. 2.1C, and Se could reduce Cd induced apoptosis.

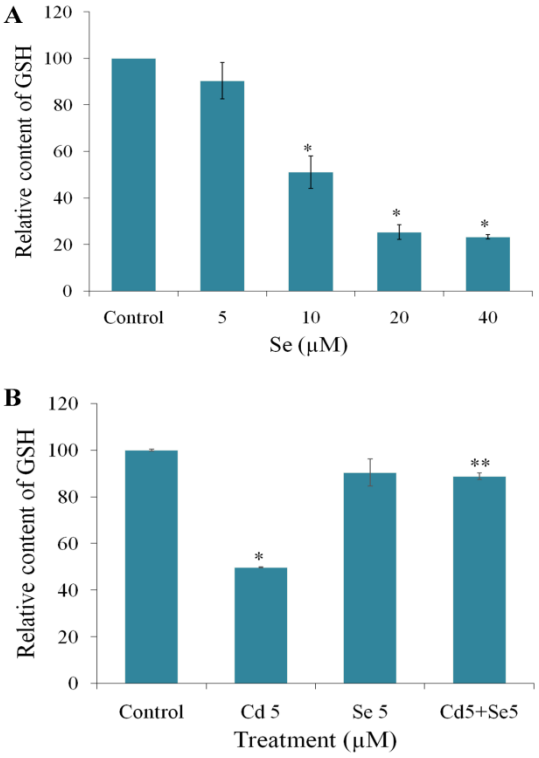

Figure 2.5: GSH (A) contents in the cultured cells treated with Se (0-40 µM); GSH (B) contents in the cultured cells treated with/without Cd (5 µM), Se (5 µM) or both metals (5 µM +5 µM) and GPx (C)contents in the cultured cells treated with Se (0-40 µM)for 48 h. Error bars are mean ± SEM (n=3). Statistical significance was evaluated by t-test: two sample assuming unequal variance, where * denotes $p < 0.05$, significantly difference from control group and ** denotes $p < 0.05$, significantly difference from only Cd-treated group.

2.3.4. Intracellular free SH levels

Reduction form of GSH, a ubiquitous tripeptidethiol, is a vital intracellular and extracellular protective antioxidant, and plays a crucial role in protecting the cells against ROS such as free radicals and peroxides (Kim et al., 2007). In ROS mediated oxidative stress, GSH is converted to GSSG. To inspect about the involvement of ROS with the toxicity of Se on PC12 cells, GSH levels were examined in the cell lysates after the treatment with Se (0, 5, 10, 20 and 40 µM). A significant decrease ($P < 0.05$) in free SH level was detected in the lysate from the cells treated with high concentrations of Se (10, 20 and 40 µM) compared to that in

the control group (Fig. 2.5A). The free SH level were decreased to 51%, 25% and 23% while the cells were treated with 10, 20 and 40 µM of Se, respectively. These results indicated that Se can generate ROS, and induces oxidative stress in PC12 cells. These findings are also in accordance with the results shown in Figs. 2.1A, 2.2 and 2.4A.

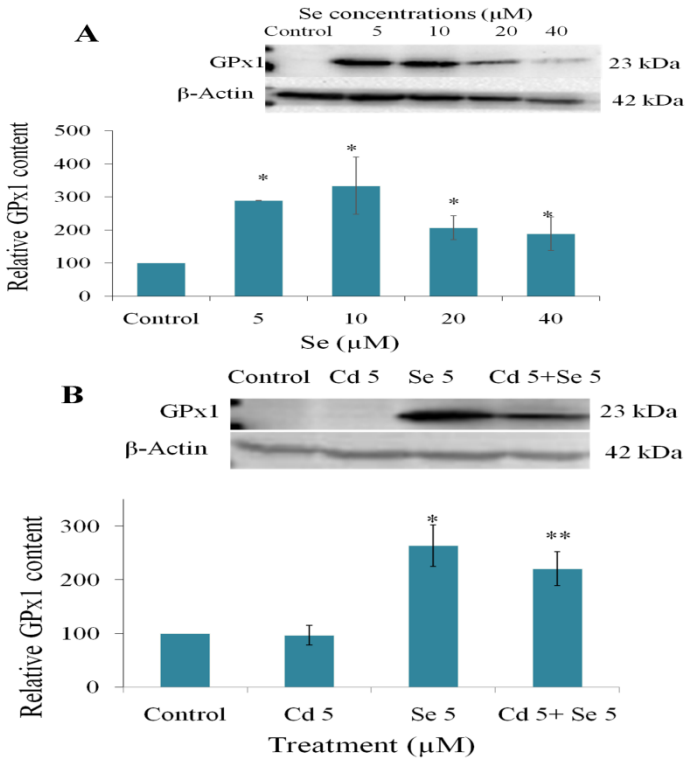

Figure 2.6: GPx contents in the cultured cells treated with Se (0–40 µM) and (B) GPx contents in the cultured cells treated with/without Cd (5 µM), Se (5 µM) or both metals (5 µM + 5 µM). Error bars are mean ± SD (n = 3). * and ** denote significantly difference ($p < 0.05$) from control group and only Cd-treated group, respectively.

The effects of Se on Cd-induced ROS generation and oxidative stress, free SH levels in the cells were measured. Fig. 2.5B shows GSH levels of the cultured cells after treatment with Cd (5 µM), Se (5 µM) or both metals (5 µM + 5 µM) for 48 h. The GSH levels were significantly decreased ($p < 0.05$) when the cells were treated with Cd (5 µM) as compared with those of control cells. However, GSH levels of cells treated with Cd (5 µM) and Se (5 µM) together were increased significantly ($p < 0.05$) as compared with those of the cells treated with only Cd. These results indicated that Cd induces ROS mediated oxidative stress in PC12 cells; however, co-exposure of Se and Cd protected the cells from Cd-induced oxidative stress. To confirm whether this antioxidant ability above mentioned is depending

on Se, GPx, a specific selenoprotein which has antioxidant property, was analyzed in the cultured cells after incubation with Se (0–40 μM) (Fig. 2.6A) and with Cd (5 μM), Se (5 μM) or both metals (5 μM+5 μM) (Fig. 2.6B) using western blotting. When the cells were treated with 5 μM of Se, it was found that GPx level was increased about three times ($p < 0.05$) compared to that in control cells. When the cells were treated with Cd (5 μM) and Se (5 μM) together, GPx level was increased more than two times ($p < 0.05$) compared to that of cells treated with only Cd (5 μM). From this result together with Fig. 2.1A, it was confirmed that Se protects the cells from Cd-induced oxidative stress by reducing ROS.

2.3.5. Protein expression analysis using western blotting

To clarify the molecular mechanism behind Se induced cytotoxicity in PC12 cells, western blot analyses of the protein extracted from PC12 cells treated with Se (0, 5, 10, 20 and 40 μM) was done evaluating the protein expression of β-Actin, caspase-3, active caspase-3, mTOR, pmTOR, p62, and ubiquitin as shown in Fig. 2.7. As shown in Fig. 2.7, there was no significant difference in expression levels of pro-apoptotic caspase-3 in PC12 cells when they were treated with different Se concentrations (0, 5, 10, 20 and 40 μM). In addition, the level of activated caspase-3 protein in PC12 cells treated with 5–40 μM of Se, did not show any significant difference as compared with that in the control cells. These results indicated that the cell death due to Se induced cytotoxicity was not caused by apoptosis process. These findings are also in accordance with the DNA fragmentation and DNA ladder pattern analyses (Fig. 2.4A).

To clarify the vital controller of cellular metabolism and proliferation and cell survival, levels of mTOR (mammalian target of rapamycin) and phosphorelated mTOR were also investigated (Fig. 2.7). Both of them showed decreasing pattern with higher Se treatment (greater than 5 μM) to the PC12 cells. The mTOR protein levels in the cells treated with 10, 20 and 40 μM of Se were significantly decreased ($p < 0.05$) compared to those in cells without treatment of Se. mTOR protein was decreased to 74%, 72% and 56%, when the cells were treated with 10, 20 and 40 μM of Se, respectively. Phosphorylated mTOR levels showed decrease tendency compared to those in cells without treatment of Se, when the PC12 cells were treated with greater than 5 μM of Se. The p62 which is associated with autophagy process was also investigated as shown in Fig. 2.7. There was a significant increase ($p < 0.05$) of p62 in the PC12 cells treated with 10, 20 and 40 μM of Se compared to those in cells without treatment of Se. Moreover, the protein level of ubiquitin which plays an important role in elongation process for autophagy were analyzed (Fig. 2.7). Expression of ubiquitin in

the PC12 cells treated with 10, 20 and 40 µM of Se, showed significant increase as compared with that in the cells without treatment of Se. From these results, it was confirmed that Se can impose toxic effects on the PC12 cells and it can induce cell death via autophagy pathway.

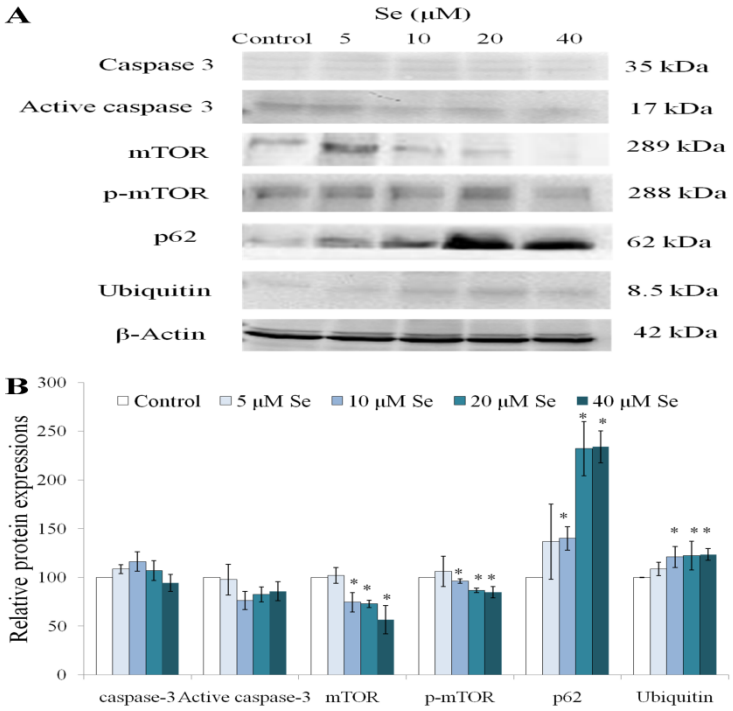

Figure 2.7: (A) Relative protein expression and (B) quantification of caspase 3, active caspase 3, mTOR, p-mTOR, p62, ubiquitin and β-actinin the cultured cells treated with Se (0-40 µM). Error bars are mean ± SEM (n=3). Statistical significance was evaluated by t-test: two sample assuming unequal variance, where * denotes $p < 0.05$, significantly different from control group.

To investigate the protection ability caused by Se on Cd-induced cytotoxicity in PC12 cells, released amounts of cytochrome c into cytosol from mitochondria in the cultured cells treated with Cd (5 µM), Se (5 µM) or both metals (5 µM + 5 µM) for 48 h were measured using western blotting (Fig. 2.8). Cytochrome c which is known to be involved in loss of mitochondrial membrane potential and initiation of apoptosis was increased significantly ($p < 0.05$) in the cytosol of the cells treated with Cd (5 µM) for 48 h as compared with that in the control cells. From this result together with DNA fragmentation analyses (Fig. 2.4B), it was confirmed that 5 µM of Cd can induce apoptosis in PC12 cells. However, contents of cytochrome c in cytosol of the cells treated with Cd (5 µM) and Se (5 µM) together were significantly reduced ($p < 0.05$) compared to those in the cells treated with only Cd (5 µM).

This result indicates that Se can reduce cytochrome c release from mitochondria, thereby protecting the cells from Cd-induced apoptosis.

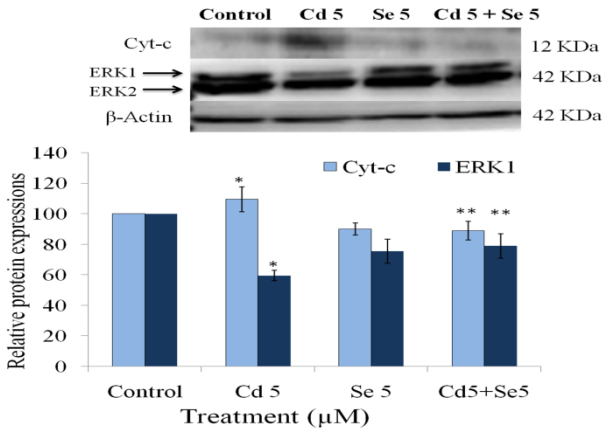

Figure 2.8: (A) Relative protein expression and (B) quantification of cytochrome c, ERK1 and β-actin in the cultured cells treated with/without Cd (5 µM), Se (5 µM) or both metals (5 µM +5 µM). Error bars are mean ± SEM (n=3). Statistical significance was evaluated by t-test: two sample assuming unequal variance, where * denotes $p < 0.05$, significantly different from control group.

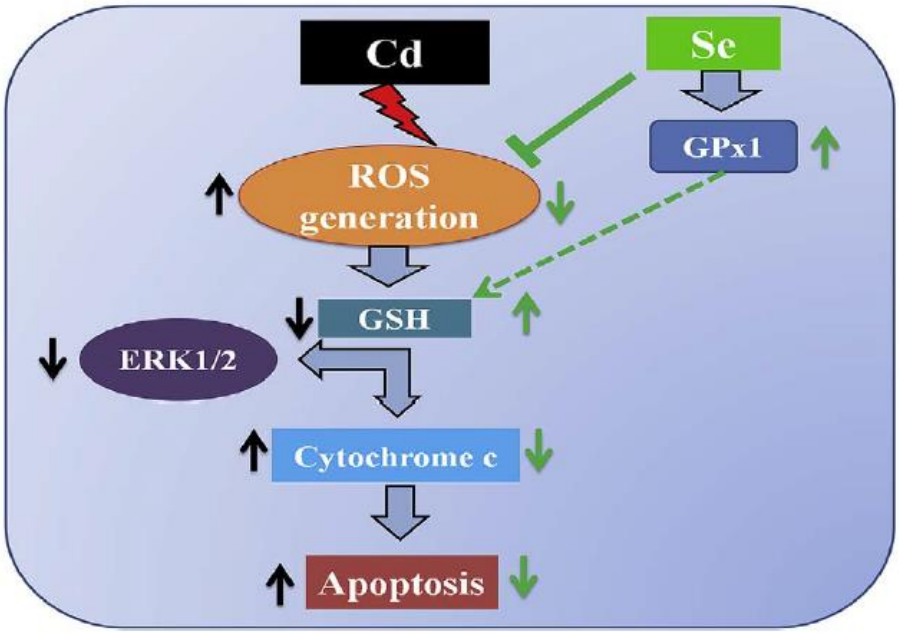

Figure 2.9: Schematic diagram of Se protection on Cd-induced cytotoxicity in PC12 cells.

Extracellular signal-regulated kinase (ERK-1), a subfamily of the classic MAP kinase family, is known as a regulator of pro-survival and anti-apoptotic protein. ERK-1 was analyzed after the cells were treated with Cd (5 µM), Se (5 µM) or both metals (5 µM + 5 µM) for 48 h (Fig. 2.8). ERK1 level was significantly decreased ($p < 0.05$) when cells were treated with Cd (5

μM) compared to that in the control cells. ERK1 level in the cells treated with Cd (5 μM) and Se (5 μM) showed significant increase ($p < 0.05$) as compared with that in the cells treated with only Cd (5 μM). It was suggested that ERK1 increased by treatment of Se to inhibit Cd-induced apoptosis. Fig. 2.9 showed a schematic diagram of Se protection on Cd-induced cytotoxicity in PC12 cells.

2.4. Discussion

It has been shown that selenium compounds could kill tumor cells with minimal side effects on normal cells (Luo et al., 2012; Huang et al., 2009; Li et al., 2013). Although cytotoxicity and anti-cancer ability of Se against multiple malignant tumors have been reported, the detailed molecular mechanisms through which these compounds exert their anti-cancer or anti-tumor effects are not yet clearly understood. However, PC12 cells which are undifferentiated cells have been used as model for brain cells upon differentiation with nerve growth factor (NGF) to clarify the mechanism of brain diseases. Precise mechanism of Se induced toxic effect on them is still unclear. In this research, the cytotoxic effects of Se on PC12 cells and the molecular mechanisms behind them were investigated. As results, it elucidates that Se can induce cytotoxicity by autophagy in PC12 cells, through inhibiting the activation of mTOR and increasing p62 and ubiquitin accumulation. These findings provide a potential approach for treatment of cancer.

Our results showed that Se exerts cytotoxicity in PC12 cells only upon high concentrations (10, 20 and 40 μM). Percentage of PC12 cell death increases with the increasing concentration of Se in the medium (Fig. 2.1A). Similar effects have been observed in the other studies using NB4 cells and various malignant glioma cells (U87MG, T98G, A172, U343 and U251) (Cummings et al., 2004; Kim et al., 2007). Half of the cells were dead within 1 and 5 μM of Se treatment in NB4 cells and glioma cells, respectively (Cummings et al., 2004; Kim et al., 2007).

The LDH activity or LDH release from the cells into the culture medium, is an indicator of membrane integrity lose or the cell-wall damage (Cummings et al., 2004). A significant increase of LDH activity in the culture medium was observed after >10 μM of Se-treatment in PC12 cells, which demonstrates the loss of cell membrane integrity (Fig. 2.2). El-Demerdash (2001) also reported that oral doses of Se increased the LDH level in serum, brain and liver of rat. In this study, an increase of DNA fragmentation in PC12 cells was observed ≥10 μM of Se exposure (Fig. 2.4A). Similarly, Zhou et al. (2003) reported that sodium selenite induced DNA damage in HL-60 leukemia cells. As the DNA fragmentation can be

induced by ROS generation (Higuchi, 2003), to estimate whether the DNA fragmentation was resulted from ROS, GSH levels which is an indicator of ROS and oxidative stress, were also measured in the PC12 cells exposed to several concentration of Se.

Oxidative stress is a complex situation regarded as an imbalance between the productions of ROS, and the availability and action of antioxidants. One of the key mechanisms by which cells protect themselves against oxidative stress is upregulation of a wide range of antioxidant genes such as GPx (Spanier et al., 2009). Reduced GSH is the most abundant intracellular non-protein thiol in cells (Zitka et al., 2012). By keeping the cellular environment in a reduced state, GSH functions in the removal of potentially toxic electrophiles and metals, thereby protecting cells from toxic ROS (Armstrong et al., 2002). It exhibits a large panel of actions in controlling gene expression, apoptosis mechanisms, or membrane transport and even DNA repair (Chatterjee, 2013). Moreover, GPx enzyme acts as a catalyst in reducing oxidized GSH and thereby protects cells from oxidative stress. In this study, the GSH levels of the cells were significantly decreased after treatment with Se (10, 20 and 40 μM) (Fig. 2.5A), whereas, GPx-1 enzyme levels were started to decrease>10 μM of Se concentration (Fig. 2.6A).

Therefore, it is proposed that 10 μM Se as a critical point of state that may induce ROS and triggers the oxidation of cellular GSH significantly; however, at the same time it enhances the GPx1 expression level. For this reason the PC12 cell viability is reduced only marginally significant ($p \leq 0.04$) with control but not significant with Se (5 μM). However, GPx1 levels in high Se concentrations (20 and 40 μM) are not capable of reducing huge amount of oxidized GSH. It means that high concentration of Se treatment caused ROS generation and oxidative stress. Increased

ROS lead to convert the reduced GSH to its oxidized form in the cell and may cause the imbalance of antioxidant and pro-oxidants in the cells. Similarly, Chen et al. (2007) reported that sodium selenite provoked oxidation of GSH with superoxide generation. Zuo et al. (2004) reported that sodium selenite exerted ROS mediated oxidative stress and the cellular GSH decrease in NB4 cells. Wallenberg et al. (2014) found that Se induced multi-targeted cell death process by ROS formation. All these reports proved that Se has a toxic effect on cells, and induces cell death.

To investigate Se-induced cell death mechanism/s in PC12 cells, protein expression analysis using western blotting is reported in this study (Fig. 2.7). Sodium selenite delivers anticancer effects through initiation of apoptosis and autophagy in several cancer cell types (Reeves and Hoffmann, 2009; Li et al., 2003). Apoptosis, type I programed cell death, also known as a

suicidal process, is characterized with distinct morphological features, such as cell shrinkage, tightly packed organelles, condensed chromatin, extensive plasma membrane blebbing, apoptotic bodies formation and finally engulfing by phagophores (Zuo et al., 2004) and Se posses the ability to induce cellular apoptosis in different cells by producing oxidative stress (Brozmanova et al., 2010).

On the other hand, autophagy is a self-degradation process which is vital for balancing sources of energy in response to nutrient/toxic stress. It has a greater variety of roles, such as starvation adaptation, intracellular protein and organelle clearance, development, anti-aging, elimination of microorganisms, cell death, tumor suppression, and antigen presentation (Mizushima, 2005). Because of the vigorous role in removing intracellular pathogens, eliminating misfolded or aggregated proteins and damaged organelles, autophagy is generally considered as a survival mechanism. However, abnormality or deregulation of autophagy has been associated to non-apoptotic cell death (Zuo et al., 2004). This process turnovers organelle and contributes in biogenic management in stress conditions and starvation (Kim et al., 2007). It is started with the formation of double-membraned structures called as autophagosomes which join with lysosomes to form autophago-lysosomes.

In autophago-lysosomes, lysosomal hydrolases break down the vesicle contents into nucleotides, amino acids, and free fatty acids that are recycled later for organelle synthesis and ATP generation. Development of autophagosomes is a three-step process characterized by nucleation, elongation, and completion of an isolation membrane or phagophore. LC3-II, p62 and ubiquitin work as structural components of the autophagosomes, and Atg proteins, including Atg12, Atg5, and Atg16L are initiators in the elongation process. Autophagy is triggered by various stimuli such as metabolic stress, energy need, and chemotherapy. Though it is considered as a survival mechanism implemented in adverse conditions to maintain cell integrity, but also involved in a death mechanism called autophagic cell death or type II cell death (Zuo et al., 2004).

A key actor in nutrient sensing and regulating cell growth, proliferation and autophagy, is well known to be the target of rapamycin (TOR) kinase (Pankiv et al., 2007). TOR kinase acts in downstream of Akt kinase, PI3-kinase and growth factor receptor, and acts to stimulate growth when nutrients are sufficient. Mammalian target of rapamycin (mTOR) functions to inhibit autophagy under such growth-endorsing conditions (Elmore, 2007). On the other hand, p62 plays an architectural role in the formation of autophagosomes and links damaged structures/organelles to the autophagic machinery via direct interaction with LC3s (Duran et al., 2011; Pankiv et al., 2007).

Interestingly, in this study it indicates that treatment with Se could effectively induce non-apoptotic and autophagic cell death in PC12 cells. It was surprisingly found that sodium selenite could trigger increase of ROS in PC12 cells, cells are trying to survive by adopting autophagy, and excessive rate of autophagy leads to cell death. Se downregulates or suppresses mTOR and/or phosphorylation of mTOR, and at the same time Se upregulates p62 and Ubiquitin (Fig. 2.7). ROS damage could cause caspase inhibition and induce autophagic cell death (Yu et al., 2006), just like the findings in this study. Kim et al. (2007) also found similar results and showed that selenite induced superoxide-mediated mitochondrial autophagic cell death in malignant glioma cells (U87MG, T98G, A172, U343 and U251). Incorporation of Se in temozolomide also showed anti-tumor activity with both apoptosis and autophagy cell death (Cheng et al., 2012).

When the PC12 cells were treated with different concentration of Se, no significant changes were found in caspase-3 and active caspase-3 protein expressions. It indicates that the cell death process is not in caspase dependent pathway (Fig. 2.7). Moreover, no ladder pattern was observed in agarose gel electrophoresis of DNA isolated from Se treated PC12 cells (Fig. 2.4A). On the basis of these results, cell death mechanism is considered other than apoptosis. mTOR, as a central controller of cell growth and autophagy in response to nutritional status, growth factor and stress signals, is reported to negatively control autophagy induction (Jung et al., 2010). In this study, it was observed that mTOR contents were significantly decreased when PC12 cells were treated with high concentrations of Se (10, 20 and 40 μM) (Fig. 2.7). Similarly, Roy et al. (2014) reported significant phosphorylated mTOR decrease in ZnO nanoparticles-induced autophagy. These results indicate the potential involvement of autophagy in PC12 cells upon Se-treatment. This observation is further supported by the significant increase of p62 and ubiquitin expressions after Se-treatment in PC12 cells (Fig. 2.7). The similar phenomenons been reported by Puissant et al. (2010). They reported the accumulation of p62/SQSTM1 in resveratrol-induced autophagic cell death in myelogenous leukemia cells (K562 and Ba/f3 p210BCR-ABL). In summary, high concentrations of Se (10, 20 and 40 μM) promote autophagy in PC12 cells through oxidative stress induction via inhibition of mTOR activation and accumulation of p62. To the best of my knowledge, this is the first report of autophagy induction in PC12 cells by Se exposure, which could be advantageous, especially in cancer cells or undifferentiated cells like as PC12 cells. While elucidating the Se-induced cytotoxicity, the antioxidant property of Se was also studied against Cd-induced toxicity in PC12 cells. In this study, it was hypothesized that Se has alleviative effects on the cytotoxicity induced by Cd in PC12 cells. Reports have already

proved that in PC12 cells Cd exhibits cytotoxicity via an extracellular ERK and JNK-mediated MAPKs and mTOR pathway via induction of ROS (Chen et al., 2008a, 2008b; Jiang et al., 2014). In PC12 cells Cd induces apoptosis via ERK, JNK mediated as well as caspase dependent intrinsic mitochondrial pathway (Jiang et al., 2014; Rahman et al., 2017) mostly by imposing oxidative stress. In this study, to clarify the protection mechanism of Se as an antioxidant on Cd-induced toxicity, the combined effects of Se under the Cd-induced apoptosis in PC12 cells were explored. The mechanism of protection by Se in PC12 cells against Cd-toxicity and the roles of ROS and ERK-MAPK pathway behind this protection were investigated. Cd induces apoptosis in various cells including PC12 cells are related to ROS generation and thereby increase of oxidative stress. As a non-fenton metal, Cd is incapable to directly induce ROS (Cuypers et al., 2010). However, Cd induces oxidative stress by; (1) a displacement of redox-active metals, (2) depletion of redox scavengers, (3) inhibition of anti-oxidant enzymes and (4) inhibition of the electron transport chain resulting in mitochondrial damage. As a thiol-affectionate metal, Cd primarily targets the highly abundant cellular GSH, a ROS scavenger (Cuypers et al., 2010). Subsequently, oxidation of GSH leads to decrease Cd-scavenging, which results imbalance of the cellular redox homeostasis by oxidative stress and ROS generation (Nair et al., 2013). In this study, it was found that 5 μM of Cd results in decrease of GSH level which exhibits increased oxidative stress (Fig. 2.5B). Increased ROS leads to convert the reduced-GSH to its oxidized form in the cell and may cause the imbalance of antioxidant and pro-oxidants in the cells. A significant reduction of GSH content have been reported by Cdexposure in eastern oysters (Ivanina et al., 2008), in HepG2 cells (Zhang et al., 2017) and also in PC12 cells (Rahman et al., 2017). It was proposed that GSH decrease may be the early and critical events for the initiation of Cd-induced apoptosis in PC12 cells.

Production of ROS in mitochondria results in reaction with mitochondrial membrane phospholipids, leading to loss of mitochondrial membrane potential, and thereby causes increased release of cytochrome c into cytosol from damaged mitochondria, activation of caspases and finally apoptosis (Fan et al., 2005). The results show 5 μM of Cd resulted in increase of oxidative stress exhibited by GSH level decrease (Fig. 2.5B), DNA fragmentation following hallmark ladder pattern (Fig. 2.4B), cell wall damage and LDH release into culture medium (Fig. 2.3), down regulation of ERK1, and increase of cytochrome c release into cytosol (Fig. 2.8), and finally induces apoptosis in the PC12 cells. Rahman et al. (2017) also reported similar results that Cd induced oxidative stress in PC12 cells by decreasing GSH level, disrupted cell membrane integrity, DNA damage and cytochrome c release from

mitochondria to cytosol. Se is known as an antioxidant and provides protection from ROS induced oxidative damage (Nishina et al., 2007). It can be available in foods and food supplements in different chemical forms. Accumulation and retention of Se in body is better with organic forms rather than inorganic forms (Finley, 2006). Organic forms of Se in foods are in reduced states (Se (−2)), whereas inorganic forms contain oxidized states (Se (+4) and Se (+6)). After absorption the higher valance containing Se forms are reduced to Se (−2) form using reduced glutathione and NADPH (Zeng, 2009). The antioxidant mechanisms for Se compounds are reported as reducing peroxides by Se-binding GPxs, scavenging of hydrogen peroxide and lipid peroxide, and metal binding mechanisms (Battin and Brumaghim, 2009). Se has regulatory roles in cell growth and survival, and cytotoxicity. El-shenawy and Hassan (2008) reported that Se can protect kidney damage induced by HgCl2 in rats. The protective effects of Se on hepatic toxicity (Newairy et al., 2007) and renal toxicity (El-Sharaky et al., 2007) induced by Cd in rats have been reported. Alleviative effect of Se on cadmium induced toxicity in chicken splenic lymphocytes (Chen et al., 2012) and lead induced toxicity in chicken testes (Wang et al., 2017) via heat shock proteins have been also reported. It is needed to conduct further study about the ameliorative effect of Se in cellular level and the molecular mechanisms involved in this protection.

To elucidate the cytoprotective mechanism of Se from Cd-induced cytotoxicity, the combined effects of Se (5 μM) and Cd (5 μM) in PC12 cells were investigated. In this study, it was found that Se protects PC12 cells from Cd-toxicity by increasing cell viability (Fig. 2.1), improving cell-wall integrity (reducing LDH release to the medium) (Fig. 2.3), reducing DNA damage (Fig. 2.4), increasing GSH level (Fig. 2.5), increasing GPx1 level (Fig. 2.6), up-regulating ERK1 and down-regulating cytochrome c release into cytosol (Fig. 2.8). These results are estimated to be depending on ROS scavenging ability of Se, as an antioxidant and may provide an effective defense from the damaging effects of Cd.

Schnabel et al. (2008) reported sodium selenite induced increased GPx1 in human coronary artery endothelial cells. Similarly, Gan et al. (2002) also reported that Se treatment might be associated with decreasing free-radical mediated lipid peroxidation, recovering GPx inhibition and generation of GSH in oxidative stress exposed PC12 cells. Chatterjee (2013) reported that increasing of GSH may also act as a modulator of the DNA-repair activity.

It was found that co-exposure of Se with Cd in PC12 cells up-regulated the ERK1, which is one of the check points to assess the activation of MAPK cascade. This result indicates the protective effects of Se on Cd-cytotoxicity may be via ERK1 and MAPK pathway. Similarly, Nishina et al. (2007) reported that ERK1 activation in Se compounds facilitated protection on

serum deprived PC12 cells. Liu et al. (2007) also reported the cytoprotective effects of Se on Cd induced apoptosis by activating JNK, which is another member of MAPK superfamily. In this study, it revealed that Se could induce limitation of DNA damage and total inhibition of cytochrome c release. In addition, Zhou et al. (2009) found that Se involved blocking of Cd induced ROS generation, inhibition of mitochondrial membrane potential impairment, and prevention of cytochrome c release. Wang et al. (2015) also reported that Se inhibited the mitochondria-mediated intrinsic apoptosis by elimination of ROS.

Finally, these results demonstrate that Se inhibits Cd-induced apoptosis in PC12 cells via increasing GPx1, limiting GSH oxidation there by restoring DNA damage, limiting LDH release and inhibiting cytochrome c release into cytosol (Fig. 2.9). Moreover, it reveals the possible involvement of ERK1- MAPK pathway. Further study is required to clarify the involvement of other pathways of MAPK. These results could help in further understanding the complex mechanisms of Cd and antioxidant activity of Se in PC12 cells, as well as other cells.

In conclusion, Se has both antioxidant and cytotoxic property on cells depending on its concentrations. It is beneficial at low concentrations to protect cells from Cd-induced toxicity, as well as, at higher concentration to induce toxicity in tumor/cancer cells to make new chemical therapeutics.

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Chapter 3: Regulatory effects of dihydrolipoic acid against inorganic mercury mediated cytotoxicity and intrinsic apoptosis in PC12 cells

Abstract

Mercury (Hg) is an extremely dangerous environmental contaminant, responsible for human diseases including neurological disorders. However, the mechanisms of inorganic Hg (iHg)-induced cell death and toxicity are little known. Dihydrolipoic acid (DHLA) is the reduced form of a naturally occurring compound lipoic acid, which act as a potent antioxidant through multiple mechanisms. So it was hypothesized that DHLA has an inhibitory role on iHg-cytotoxicity. The purposes of this research were to investigate mechanism/s of cytotoxicity of iHg, as well as, the cyto-protection of DHLA against iHg induced toxicity using PC12 cells. Treatment of PC12 cells with $HgCl_2$ (Hg^{2+}) (0-2.5 µM) for 48 h resulted in significant toxic effects, such as, cell viability loss, high level of lactate dehydrogenase (LDH) release, DNA damage, cellular glutathione (GSH) level decrease and increased Hg accumulation. In addition, protein level expressions of akt, p-akt, mTOR, GR, NFkB, ERK1, Nrf2 and HO-1 in cells were downregulated; and cleaved caspase 3 and cytochrome c release were upregulated after Hg^{2+} (2.5 µM) exposure and thus inducing apoptosis. However, pretreatment with DHLA (50 µM) for 3 h before Hg^{2+} (2.5 µM) exposure showed inhibition against iHg^{2+}-induced cytotoxicity by reversing cell viability loss, LDH release, DNA damage, GSH decrease and inhibiting Hg accumulation. Moreover, DHLA pretreatment reversed the protein level expressions of akt, p-akt, mTOR, GR, NFkB, ERK1, Nrf2, HO-1, cleaved caspase 3 and cytochrome c. In conclusion, results showed that DHLA could attenuate Hg^{2+}-induced cytotoxicity via boosting up of antioxidant defense, limiting Hg accumulation and inhibition of apoptosis in cells.

3.1. Introduction

Alpha lipoic acid (α-LA) and its reduced form dihydrolipoic acid (DHLA) are thiol containing natural antioxidants synthesized enzymatically in mitochondria. Lipoic acids can pass through blood-brain barrier and well known as a universal antioxidant, although it has controversy. Studied antioxidant properties of lipoic acid are: metal chelation, reactive oxygen species (ROS) scavenging, recycling or regeneration of other endogenous antioxidants and oxidative damage repair. DHLA is considered to have more antioxidant properties than LA, because both have properties of metal chelation and ROS scavenging, but DHLA only has the properties of endogenous antioxidants regeneration and repairing

oxidative damage (Smith et al., 2004; Bjorklund et al., 2019). LA is produced de novo by lipoic acid synthase in mammalian mitochondria (Packer et al., 1995). But LA can also be accumulated from food intake and rapidly converted to DHLA in many tissues (Packer et al., 1995). High amount of LA containing foods are red meat (muscle meats, heart, kidney and liver), spinach, carrots, broccoli, potatoes, yams, tomatoes, yeast, brussels sprouts, beets, and rice bran etc (Shay et al., 2009). Though available from these normal nutritional sources, dietary supplement of LA is also renowned. Several lines of studies have already established that LA provides protection counter to alteration initiated by oxidative stress triggered by toxic substances or heavy metals (El-Maraghy et al., 2011; Tahira et al., 2012).

Heavy metal Mercury (Hg) is an enormously hazardous environmental pollutant, which is liable for exposure and toxicity induction in human. By the report of Agency for Toxic Substances and Disease Registry (ATSDR), Hg is the third, after arsenic (As) and lead (Pb) among most hazardous substances. Human can be exposed to Hg in both of its inorganic and organic compounds. Elemental Hg vapor is accumulated through inhalation. Organic Hg source includes consumption of seafood or from medical products and dental amalgam is a source of inorganic Hg. Hg easily crosses blood brain barrier and causes detrimental consequences on central nervous system. Even a low dose of Hg causes neural system disruption, memory loss, Alzheimer like dementia, motor dysfunction, decreased fertility, abnormality in offspring, autism, decreased immunity, sensory disturbances, impairment in hearing and vision, increased fatigue, renal and cardiovascular dysfunction (Kim and Sharma, 2003; Rahman et al, 2019).

Recently, in vivo (Yang et al. 2016a; Morgan et al., 2006) and in vitro (Yang et al. 2016b; Yin et al., 2007; Petroni et al., 2013) studies explored Hg-cytotoxicity. Though, little was known of the interaction and molecular mechanisms responsible for Hg-toxicity. Several studies have reported that Hg generates reactive oxygen species (ROS) and induces oxidative stress in vivo and in vitro (Kim and Sharma, 2003; Yang et al. 2016a, 2016b).

Natural products and their derivatives have shown cytoprotective effects against heavy metals induced toxicity (Sobral-Souza et al. 2019a, 2019b; Rocha et al. 2018, 2019). In present research, natural product DHLA was used to evaluate iHg-cytotoxicity and to examine the cytoprotective role of DHLA against iHg induced cytotoxicity *in vitro*. Recently the effects upon pre-exposure or simultaneous exposure of antioxidant substances with heavy metal have been assessed in various cell lines. For example, selenium protects Sp1K cells from Hg-toxicity (Wang et al., 2001); LA serves protection on Cd-influenced oxidative stress and toxicity in HepG2 cells (Shi et al., 2016). In addition, LA-induced protection on Hg-toxicity

has been studied *in vivo* (Yang et al. 2015, 2016a) and *in vitro* (Hultberg, 2002; Yang et al. 2016b). However, most of the studies were concentrated on the effect of organic Hg, whereas the effect of iHg has been unexplored. And the investigation about the effects of reduced form of LA i.e DHLA against iHg toxicity is still unexplored. Moreover, the precise biomolecular mechanism/s of this protection is inadequate and needs further investigation. Therefore, in this research, for the very first time, the role of DHLA on iHg-induced toxicity was explored in PC12 cells. Here, it was hypothesized that DHLA has protective effects against iHg-cytotoxicity in PC12 cells. Therefore, to investigate this hypothesis several cytotoxicity evaluation methods were used following exposure of PC12 cells to iHg with/without DHLA-pretreatment for a definite incubation time; for instance, cell viability assay, lactate dehydrogenase activity assay, DNA fragmentation assay, glutathione (GSH) contents assay, apoptosis assay using flow cytometry, western blotting analysis for protein expression and Hg accumulation in cells by reduction vapor atomic absorption spectrophotometry.

3.2. Methodology

3.2.1. Cell culture

DMEM (Sigma, USA) supplemented with FBS (10%) (Biosera, USA) in a humidified incubator was used for cell culture. The PC12 cells were cultured at 37°C with 5% CO_2. After 48h, the medium was substituted with DMEM with/without concentrations of $HgCl_2$ (Hg^{2+}) (0.65, 1.25, 2.50, and 5 μM) as a source of inorganic Hg and DHLA (0, 25, 50, 100, and 150 μM). Best combinations conducive to cell growth were selected. The chosen concentrations for Hg^{2+} were 1.25 and 2.50 μM. DHLA (50 μM) was used as pretreatment 3h prior to the Hg treatment to the cells and incubated for 48 h. DHLA and $HgCl_2$ were obtained from Tokyo Chemical Industries Co. and Wako Pure Chemical Corporation (Japan), respectively. Thus, PC12 cells incubated with/without Hg^{2+} (0, 1.25, and 2.50 μM) and with/without DHLA (0, 50 μM) in a factorial treatment matrix were incubated for 48 h, and then subjected to assessments for cell viability, LDH activity, quantification and extraction of DNA, SH levels, apoptosis, western blot and elemental analysis.

3.2.2 Viability of the cells

A trypan blue exclusion test was operated for analyzing viability of the cells. PC12 cells were preincubated in DMEM supplemented with 10% FBS for 48 h. After that, the cells were incubated with and without Hg^{2+} and DHLA pretreatment. An automated cell counter (Bio-

Rad, USA) was used to measure trypan blue (Bio-Rad, USA)-stained and non-strained cells after 48 h of incubation. Each experiment was performed more than 10 times to verify reliability.

3.2.3. Lactate dehydrogenase (LDH) activity analysis

Membrane integrity of the cells following treatment with $HgCl_2$ was assessed by measuring LDH activity, by cytotoxicity kit (Promega, USA). PC12 cells were incubated with/without Hg^{2+} (0, 1.25, and 2.50 μM), DHLA (50 μM), and combinations of DHLA pre-treatment and Hg^{2+} for 48 h. After incubation, the medium (50 μL) of the cells was used to measure LDH activity, as described in previous research (Hossain et al. 2018). These assays were executed four times to justify reproducibility.

3.2.4. Extraction of genomic DNA from cells

Cells were treated with/without Hg^{2+} (0, 1.25, and 2.50 μM), DHLA (50 μM), and combinations of DHLA pre-treatment and Hg^{2+}; and after 48 h the cells were harvested, washed with 1x PBS and the genomic DNA was extracted using High Pure PCR Template preparation kit (Germany) as explained by Hossain et al. (2018).

3.2.5. Electrophoresis of genomic DNA

Agarose gel electrophoresis analysis was used to observe the amount of intact DNA. 5 μM of isolated DNA of the cells exposed to Hg^{2+} (0, 1.25, and 2.50 μM), DHLA (50 μM) and combinations of DHLA pre-treatment and Hg^{2+} for 48 h were analyzed by electrophoresis with a 1.5% agarose gel as dexplained by Rahman et al. (2018). Quantity One™ (Bio-Rad, USA) software was used to assess the amount of intact DNA. This process was replicated five times to verify reliability.

3.2.6. Intracellular free sulfhydryl (SH) levels assay

To investigate free SH in intracellular level, cells were exposed with Hg^{2+} (0, 1.25, and 2.50 μM), DHLA (50 μM) and combinations of DHLA pretreatment and Hg^{2+}. After 48 h of incubation the cells were harvested, washed with ice cold 1x PBS, lysis buffer (150 μL) was added, and the sample was kept for 10 min at 4 °C. Two cycles of freeze-thawing and sonication were employed to break the cell membranes. Then the solution was centrifuged at 1500 rpm for 10 min, the supernatant were collected and the protein content of it was determined spectrophotometrically. After that free SH of the cell supernatants were measured, as described by Hossain et al. (2018) and Rahman et al. (2017). The tests were conducted three times to confirm instrumental reproducibility.

3.2.7. Flow cytometry for apoptosis detection

Cell death process in PC12 cells was analyzed by flow cytometry with Annexin V-FITC apoptosis detection kit (BioVision, USA). PC12 cells were exposed to Hg^{2+} (0, and 2.50 μM), DHLA (50 μM) and combinations of DHLA pretreatment and Hg^{2+}; and incubated for 48 h. Then the cells were harvested and approximately 1-5 × 10^5 cells were washed with 1x PBS and centrifuged. Then cells were suspended in 1x binding buffer (500 μL). Annexin V-FITC (5 μL) and propidium iodide (5 μL) were added and retained for 5 min in the dark at room temperature. Cells were then analyzed using BD FACSVerse™ flowcytometer (BD Biosciences). These tests were performed in triplicate to verify reproducibility.

3.2.8. Western blot for protein expression determination

PC12 cells were incubated in DMEM containing 10% FBS with/without Hg^{2+} (2.50 μM), DHLA (50 μM) and, combinations of DHLA pretreatment and Hg^{2+}. The cells were harvested after 48 h of incubation and ice-cold PBS was added into the cells. Protein extraction from the cells and electrophoresis were performed as described in previous research (Hossain et al. 2018). Those electrophoresed proteins were shifted to a nitrocellulose membrane using a semidry blotting system (Japan). The proteins in the membranes were blocked using skimmed milk, washed three times with washing buffer and kept overnight with the primary antibodies at 4°C. Polyclonal antibodies against β-actin (4967, Cell Signalling Technology), glutathione reductase (ab16801, Abcam), akt (4691, Cell Signalling Technology), p-akt (9267, Cell Signalling Technology), mTOR (2972, Cell Signalling Technology), p-mTOR (Cosmo Bio Co.), Nrf2 (PM069, Medical and Biological Laboratories Co., Japan), Heme oxygenase 1 (GTX101147, GeneTex), ERK1 (610031, BD Transduction Laboratories), NFkB p65 (D14E12, Cell Signalling Technology), IkBα (4814, Cell Signalling Technology). Phosphorylated-IkBα (2859, Cell Signalling Technology), caspase-3 (GTX110543, GeneTEX), cleaved caspase-3 (9661, Cell Signalling Technology), LC3B (83506, Cell signaling Technology) and cytochrome c releasing apoptosis kit (Calbiochem®) were used. Following day membranes were washed, incubated with the secondary antibody on a shaker for 1h and then washed for 5 times. The protein bands were detected by enhanced chemiluminescence and later the detected bands were studied by a ChemiDoc XRS (Bio-Rad, USA). Experiments were done at least four times to verify reproducibility.

3.2.9. Elemental analysis of inorganic mercury concentration

Cells were incubated in medium with/without Hg^{2+} (2.50 μM), DHLA (50 μM) and, combinations of DHLA pretreatment and Hg^{2+}. The cells were harvested after 48 h of

incubation and 5 mL of PBS was added into the cells. The mixture was centrifuged (1500 rpm) and the supernatants were separated. Cells and culture media were digested with sulfuric acid and potassium permanganate. After decomposition of the samples, Hg contents were measured by reduction vapor AAS using a Direct Thermal Decomposition Mercury Analyzer MA-3000 with reducing-vaporization-atomic absorption measurement S-MA (Nippon Instrument Corporation, Japan).

3.2.10. Statistical analysis

Data were represented as the mean ± standard error of mean (SEM). Single-factor analysis of variance (ANOVA), followed by unpaired Student's t-test were employed to conduct statistical analysis. Results were considered significantly different at $p < 0.05$ or $p < 0.01$.

3.3. Results

3.3.1. Cell Viability Measurement

To assess the effects of DHLA, PC12 cells were exposed to 0, 25, 50, 100 and 150 µM of DHLA. Fig 3.1A displays the cell viability of PC12 cells 48h following incubation with DHLA. The cell viability was not changed due to DHLA exposures (25, 50, 100 and 150 µM) for 48 h. This study indicated that the concentrations of DHLA upto 150 µM were not harmful for PC12 cells.

Again, trypan blue straining assay was used to analyze the cell viability of the combined treatment. Concentrations of iHg were selected based on dose dependency of iHg toxicity and LD_{50} value (2.39 µM) of iHg. And DHLA concentration has been chosen depending on the lowest concentration of DHLA to provide approximately 100% protection from iHg induced toxicity. Fig. 3.1B showed the viability of PC12 cells exposed to Hg^{2+} (0, 1.25 and 2.5 µM), DHLA (50 µM) and combinations of Hg^{2+} and DHLA for 48 h. It revealed that the viability of cells exposed to Hg^{2+} concentrations (1.25 and 2.5 µM) was decreased significantly ($p<0.05$) from that of the control group. Though, viability test of the cells treated with only 50 µM of DHLA was not significantly different from the control; the cell viability of cells pretreated with DHLA (50 µM) 3h prior to the exposure of Hg^{2+} (1.25 or 2.5 µM) was significantly ($p<0.05$) increased from that of the cells treated with only iHg concentrations (1.25 and 2.5 µM). These data recommend that DHLA has protective effect on Hg-induced toxicity in PC12 cells.

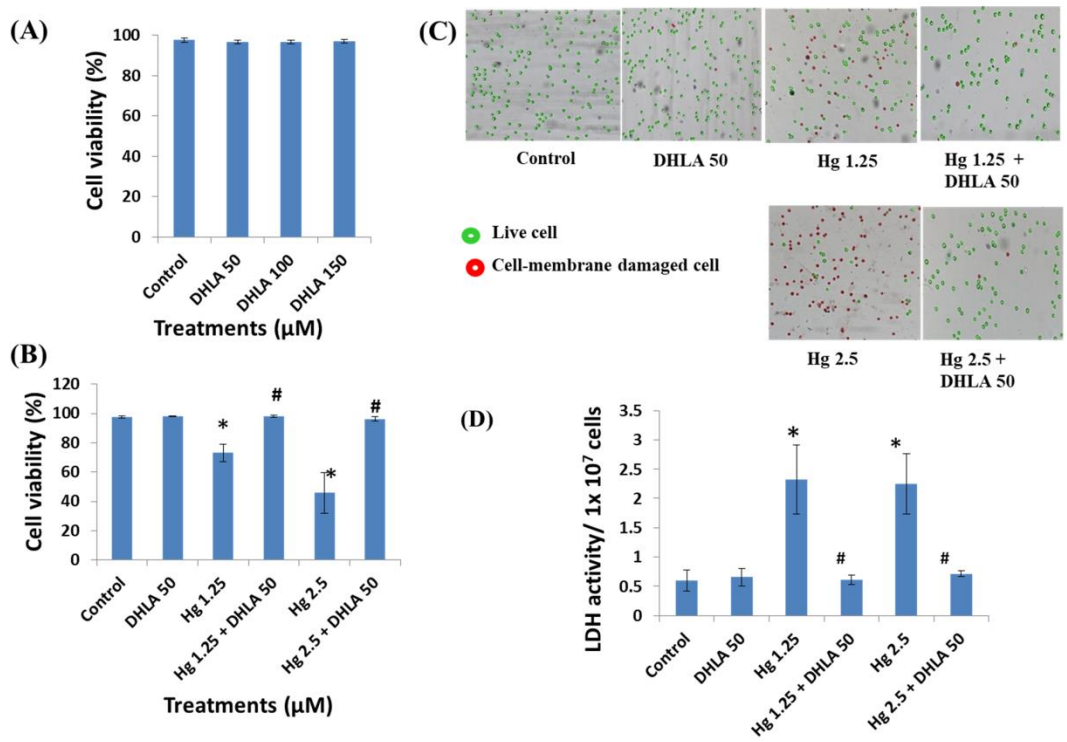

Figure 3.1: (A) Viability of PC12 cells treated with 0-150 µM of DHLA, (B) Viability of PC12 cells after Hg (1.25 and 2.5 µM) exposure, with/without pretreatment of DHLA (50µM). (C) Cell membrane damage observation in PC12 cells treated with/without DHLA (50µM) and Hg (1.25 and 2.5 µM) for 48 h. (D) LDH levels in the culture media of PC12 cells exposed with/without DHLA (50µM) and Hg (1.25 and 2.5 µM) for 48 h. Each column is shown as mean ± SEM, ((A) n=4, (B) n=6, (C) n=4 and (D) n=4). * and # denotes significantly different value ($p < 0.05$) from control and only Hg-treated cells of relative concentrations, respectively.

3.3.2. Cell-Membrane Damage

The observation of the cell-membrane damage in PC12 cells was obtained by performing cell staining with trypan blue and LDH release assay. Fig 3.1C showed the picture of PC12 cells strained with trypan blue. Red color indicating dead cells were increased significantly when treated with Hg^{2+} (1.25 and 2.5 µM) compared to those in the control cells (Fig 3.1C). The membrane-damaged cells (red colored cells) were significantly reduced in group pretreated with DHLA (50 µM) and then exposed to Hg^{2+} (1.25 and 2.5 µM), compared to the cells exposed to only Hg^{2+} concentrations (1.25 and 2.5 µM) without pretreatment.

To verify the ameliorative effects of DHLA pretreatment in Hg-induced cell membrane damage, LDH activity of the PC12 cells was examined (Fig 3.1D). The cell culture medium

of the group exposed to only Hg^{2+} concentrations (1.25 and 2.5 µM) showed a significantly increase ($p<0.05$) in LDH levels from that of the media of control group. It was suggested that Hg^{2+} concentrations (1.25 and 2.5 µM) provided lethal effects on cells and caused loss of membrane integrity. Conversely, cells pretreated with DHLA before Hg^{2+} exposure showed significantly reduced LDH levels. These results suggested that DHLA can limit cell membrane integrity lose induced by Hg^{2+}. These observations exhibited the similar trend with the cell viability (Fig 3.1B and Fig 3.1C).

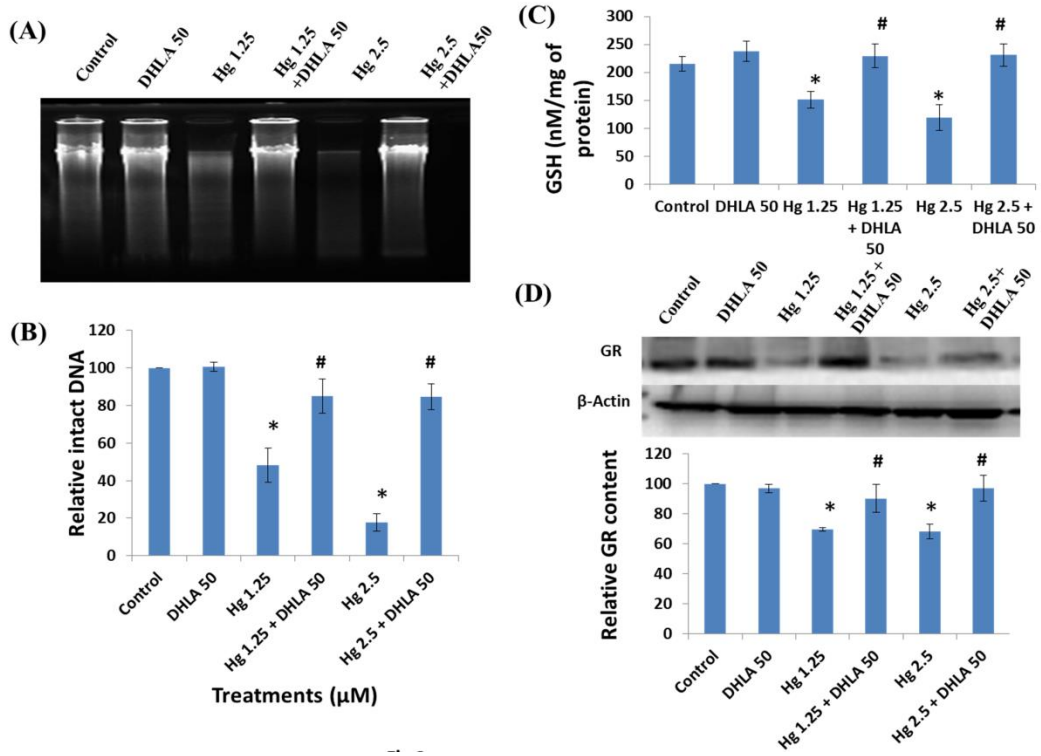

Figure 3.2: (A) Agarose gel electrophoresis of DNA isolated from PC12 cells treated with/without DHLA (50µM) and Hg (1.25 and 2.5 µM) for 48 h. (B) Intensity of DNA in the electrophoresis band. (C) GSH contents and (D) glutathione reductase (GR) expressions of the cells treated with/without DHLA (50µM) and Hg (1.25 and 2.5 µM) for 48 h. Error bars are mean ± SEM, ((A and B) n=5, (C) n=3, and (D) n=4). * and # denotes significantly different value ($p < 0.05$) from control cells and only Hg-treated cells of relative concentrations, respectively.

3.3.3. DNA degradation

The DHLA-amelioration on Hg^{2+} influenced DNA degradation was observed by extraction of genomic DNA from treated PC12 cells and electrophoresis. Fig. 3.2A and 2B show the image of electrophoresis and intensity quantification of DNA isolated from cells exposed to Hg^{2+}

(1.25 and 2.5 μM) with/without pretreatment of DHLA (50 μM) for 48 h. The relative intact DNA amount was decreased significantly when PC12 cells was exposed to Hg^{2+} concentrations only (1.25 and 2.5 μM); however, the relative intact DNA amount was significantly increased ($p<0.05$) when PC12 cells received DHLA pretreatment before Hg^{2+} exposures. These findings indicated that a significant DNA damage induced by Hg^{2+} concentrations was blocked due to pretreatment of DHLA. These findings support the results from cell viability analysis, and suggest that DHLA pretreatment could inhibit Hg^{2+} induced DNA damage.

3.3.4. Intracellular GSH levels

Reduced form of glutathione (GSH) is a crucial antioxidant, which acts an essential job in defending the cells from oxidative damage (Kim et al., 2007). GSH is converted to GSSG in ROS mediated stress. The involvement of ROS in Hg^{2+}-toxicity and the effects of DHLA pretreatment on Hg^{2+}-influenced oxidative stress and ROS production, reduced glutathione or free SH levels were investigated. Fig. 3.2C displays GSH content of the cells following exposure to Hg^{2+} (1.25 or 2.5 μM), with/without pretreatment of DHLA (50 μM). As shown in the Fig. 3.2C, The GSH contents were decreased significantly ($p<0.05$) in the cells exposed to Hg^{2+} concentrations (1.25 or 2.5 μM) from that of the control cells. On the contrary, cells pretreated with DHLA (50 μM) before Hg^{2+} exposure (1.25 or 2.5 μM) showed significantly improved ($p<0.05$) GSH contents from that of the cells treated with only Hg^{2+} (1.25 or 2.5 μM). These findings suggested that Hg persuades ROS arbitrated oxidative stress in PC12 cells, but pretreatment with DHLA before Hg^{2+} exposure, inhibited Hg-induced oxidative stress in the cells.

Glutathione reductase (GR), which is a ubiquitous enzyme, vital for the redox cycle of glutathione, conserves reduced GSH in cells in a sufficient level of and thereby recycles cellular antioxidant. GR protein expression was significantly ($p<0.05$) down regulated in cells exposed to Hg^{2+} (1.25 and 2.5 μM) concentrations alone, suggesting a significant reduction in antioxidant contents from that of the control group (Fig 3.2D). Conversely, as compared with cells that were treated with Hg^{2+} concentrations (1.25 and 2.5 μM) alone, the DHLA-pretreated groups showed a significant ($p<0.05$) upregulation of GR expression. These results confirmed that DHLA pretreatment shields the cells from Hg-influenced oxidative stress by dropping ROS and increasing reduced glutathione in the cells. As the results of Hg^{2+} (1.25 and 2.5 μM) treatments in cells are showing similar trends, the highest concentration of Hg^{2+} (2.5 μM) has been analyzed afterwards.

3.3.5. Flow cytometry analysis for apoptosis detection

The type of iHg-influenced cell death and the protection of DHLA pretreatment was confirmed using flow cytometry where PC12 cells were exposed to Hg^{2+} (2.5 µM) with/without DHLA pretreatment (Fig 3.3). Treatment of cells with 2.5 µM of Hg^{2+} for 48h increased the apoptotic cells, where pretreatment of cells with DHLA (50 µM) before Hg-exposure showed a significant decrease in apoptotic cells.

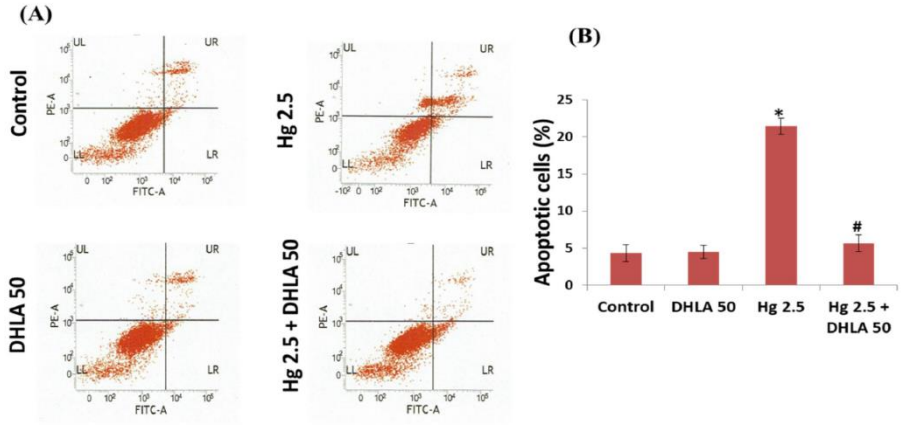

Figure 3.3: (A) Flow cytometry of cultured PC12 cells, (strained with Annexin V-FITC/PI) following exposure with/without DHLA (50µM) and Hg (2.5 µM) for 48 h and (B) percentage of apoptotic cells. Error bars are mean ± SEM, (n=3). * and # denotes significantly different value ($p < 0.05$) from control group and only Hg-treated group, respectively.

3.3.6. Protein expressions of the cells

To explore molecular mechanisms of DHLA pretreatment on Hg-influenced toxicity in PC12 cells, western blotting was conducted from the protein extracts of PC12 cells exposed with/without Hg^{2+} (2.5 µM) and DHLA pretreatment (50 µM), assessing the expressions of β-Actin, mTOR, p-mTOR, akt, p-akt, Nrf2, HO-1, NFkB, IkBα, p-IkBα, ERK1 caspase-3, active caspase-3, cytochrome c, and LC3 as shown in fig 3.4 and fig 3.5.

The levels of mTOR (mammalian target of rapamycin), the key controller of metabolism, proliferation and survival of the cell, was investigated (Fig 3.4). mTOR protein was significantly ($p<0.05$) decreased in the cells exposed to Hg^{2+} (2.5 µM); and DHLA pretreatment displayed a significant ($p<0.05$) upregulation of m-TOR protein expression, though no significant difference was found in p-mTOR protein expression. A vital player in transcription and cell migration, glucose metabolism, cell proliferation and apoptosis, the protein level of Akt and its phosphorelated form were also investigated. Both of them showed significant ($p<0.05$) decreasing pattern with Hg^{2+} treatment (2.5 µM) to the PC12 cells. The

akt and p-akt protein levels in the Hg^{2+} (2.5 µM) treated cells with pretreatment of DHLA (50 µM), were upregulated significantly ($p<0.05$) from those in cells without pretreatment.

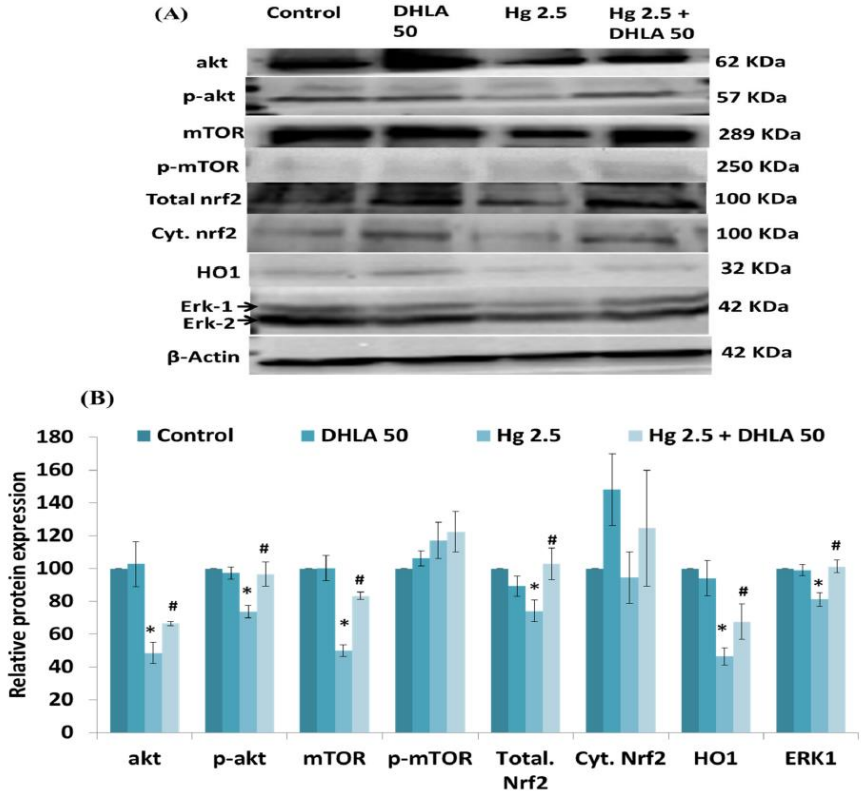

Figure 3.4: (A) Relative protein expression and (B) estimation of akt, p-akt, mTOR, p-mTOR, Nrf2, HO-1, ERK1 and β-actin in the PC12 cells exposed with/without DHLA-pretreatment (50µM) and Hg (2.5 µM) for 48 h. Error bars are mean ± SEM (n= atleast 4). * and # denotes significantly different value ($p < 0.05$) from control cells and only Hg-treated cells, respectively.

Nrf2 is a cytoprotective protein, which regulates genes expression for antioxidant, anti-inflammatory and detoxifying proteins by inducing GR and heme oxygenase-1 (HO-1); and HO-1 exerts protection from oxidative injury, apoptosis and inflammation, which is regulated through Nrf2. To evaluate the involvement of nrf2 and activation of HO1, the protein expressions were analyzed. Total Nrf2 and HO1 protein expressions were significantly ($p<0.05$) reduced upon Hg^{2+} (2.5 µM) exposure when compared with control group and pretreatment of DHLA showed a significant ($p<0.05$) upregulation in both total nrf2 and HO1 expression (Fig 3.4). Cytoplasmic nrf2 showed an increase in both groups treated with only DHLA and DHLA pretreated Hg^{2+}-treatment group, though those are not significant. These results suggested that Nrf2 and HO1 were also involved in DHLA protection to Hg-induced toxicity.

A subfamily of MAP-kinase family, ERK-1, is a controller of cell survival and apoptosis regulation. Expression of ERK-1 was studied from protein extract of the cells treated with/without Hg^{2+} (2.5 µM) and DHLA (50 µM) for 48 h (Fig 3.4). Expression of ERK1 was significantly decreased ($p<0.05$) in cells exposed to Hg^{2+} (2.5 µM) from those of the control cells. ERK1 expression of the cells pretreated with DHLA (50 µM) before Hg^{2+} (2.5 µM) treatment revealed significant increase ($p<0.05$) from the cells exposed with only Hg^{2+} (2.5 µM). It was indicated that ERK1 increased by DHLA-pretreatment inhibits Hg-induced apoptosis.

A protein complex which regulates DNA transcription, production of cytokine and cell survival is NFkB. As shown in fig 3.5, expressions of anti-apoptotic protein NFkB was significantly ($p<0.05$) decreased by Hg^{2+} (2.5 µM), however pretreatment of DHLA (50 µM) significantly ($p<0.05$) upregulated the NFkB protein expression compared to the group treated with only Hg^{2+} (2.5 µM). IkBα is a member from a family of cellular proteins that act as an inhibitor of NFkB transcription factor. There was no significant change in IkBα and p-IkBα protein expression in PC12 cells upon DHLA or Hg exposure.

To verify the molecular mechanism of Hg-cytotoxicity in cells and to examine the effect of DHLA pretreatment on this cytotoxicity, western blot analyses were done examining the pro-apoptotic protein expressions of caspase-3, cleaved caspase-3 and cytochrome c as shown in fig 3.5. Caspase-3 expression was significantly ($p<0.05$) reduced in cells exposed to Hg^{2+} (2.5 µM) from the control cells. However, Caspase-3 protein expression was significantly ($p<0.05$) upregulated when cells were pretreated with DHLA before Hg^{2+} treatment. Cleaved or activated caspase-3 expression was significantly ($p<0.05$) increased in only- Hg^{2+}-treated cells, but was significantly ($p<0.05$) reduced in cells pretreated with DHLA. Similarly, cytochrome c, well-known for its involvement in membrane potential loss of mitochondria and apoptosis induction, was upregulated ($p<0.05$) significantly in the cytosol, when the cells were treated with Hg^{2+} (2.5 µM) compared to the control cells. From this findings it was confirmed that 2.5 µM of Hg^{2+} induces intrinsic apoptosis in PC12 cells. In contrast, cytosolic cytochrome c level of the cells pretreated with DHLA (50 µM) and treated with Hg^{2+} (2.5 µM), were downregulated ($p<0.05$) significantly compared to the cells exposed to only Hg^{2+} (2.5 µM). These data indicate that DHLA can prevent cytosolic cytochrome c and thus protecting the cells from Hg-induced intrinsic apoptosis.

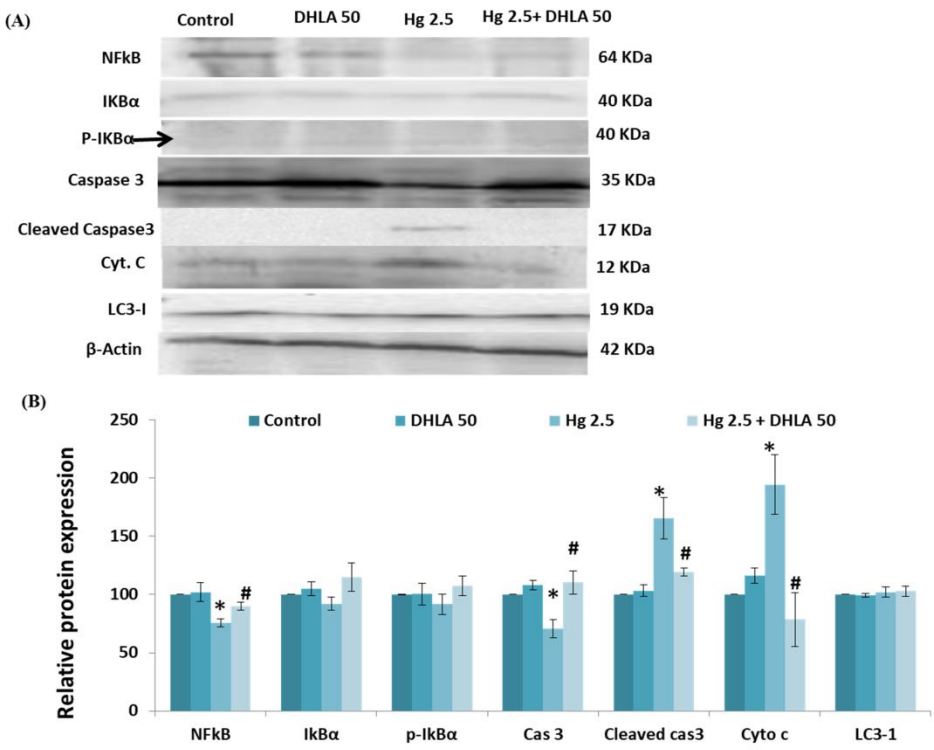

Figure 3.5: (A) Relative protein expression and (B) estimation of NFkB, IkBα, p- IkBα, caspase 3, cleaved caspase 3, cytochrome c, LC3-I and β-actin in the PC12 cells exposed with/without DHLA (50μM) and Hg (2.5 μM) for 48 h. Error bars are mean ± SEM (n=atleast 4). * and # denotes significantly different value ($p < 0.05$) from control cells and only Hg-treated cells, respectively.

LC3 or 1A/1B-light chain 3, which is a vital protein in autophagy, where LC3-I is transformed into LC3-II to form autophagosomes, was also analyzed to investigate the involvement of autophagy type of cell death (Fig 3.5). Though no significant difference was found in LC3-I and no LC3-II protein expressions was found in PC12 cells, which prove autophagy was not involved in both Hg^{2+}-induced cell death and DHLA-protected cell survival process in PC12 cells.

However, upon pretreatment of DHLA (50 μM) showed significant inhibitory effects against Hg^{2+} (2.5 μM) on the regulation of apoptotic proteins. These findings suggested that DHLA (50 μM) pretreatment impeded Hg^{2+} (2.5 μM)-induced apoptosis in PC12 cells.

3.3.7. Determination of Hg concentrations

To explore the iHg accumulation into the cells, the concentrations of total Hg concentration was investigated in cells and also in culture media. Fig. 3.6 shows Hg concentrations in (A) the PC12 cells (ng/ $1x10^6$ cells) and (B) culture media (μM) of the cells. The concentrations

of Hg in the control cells and control culture media were below the detectable level, because of insignificant presence of Hg in those groups. The cells treated with only Hg (2.5 µM) showed a significant ($p<0.05$) amount of Hg in cell digest (Fig. 3.6(A)), indicating cellular accumulation while compared with the control group. Again, the DHLA pretreated cells demonstrated a significant ($p<0.05$) drop (approximately 2/3) of the Hg amount compared to the cells treated with only-Hg (Fig. 3.6(A)), indicating lower accumulation of iHg in presence of DHLA, which also indicates a possibility of chelation between iHg and DHLA in the culture media. Again in Fig. 3.6 (B), the cells treated in Hg^{2+} (2.5 µM) with/without DHLA-pretreatment showed a significant increase of Hg concentration in the culture media compared to the control group cells. However, the concentration of Hg in the culture media did not express any significant difference between the groups treated with only-Hg and the DHLA pretreated Hg-treatment, which also support the idea of chelate formation of Hg with DHLA in the culture media.

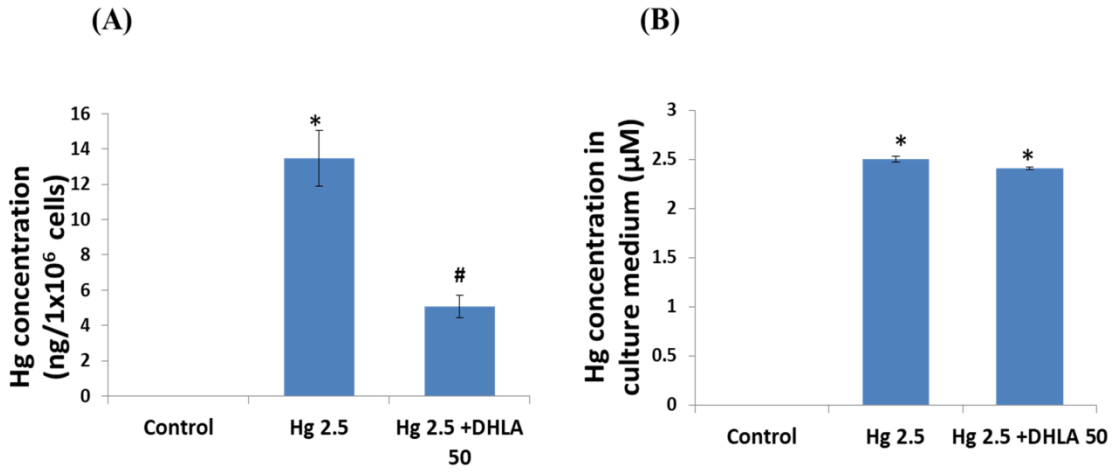

Figure 3.6: Total Hg concentrations in (A) the PC12 cells and (B) the culture media (µM) of the cells exposed with/without DHLA (50 µM) and Hg (2.5 µM) for 48 h. Error bars are mean ± SEM (n=3). # denotes significant difference ($p < 0.05$) from only-Hg-treated group.

From these findings, chelate formation was indicated as the major phenomena responsible for DHLA-protection on iHg induced toxicity in PC12 cells with other antioxidant properties. A simplified illustration of regulatory effects of DHLA against iHg-induced cytotoxicity in PC12 cells was shown in Fig. 3.7.

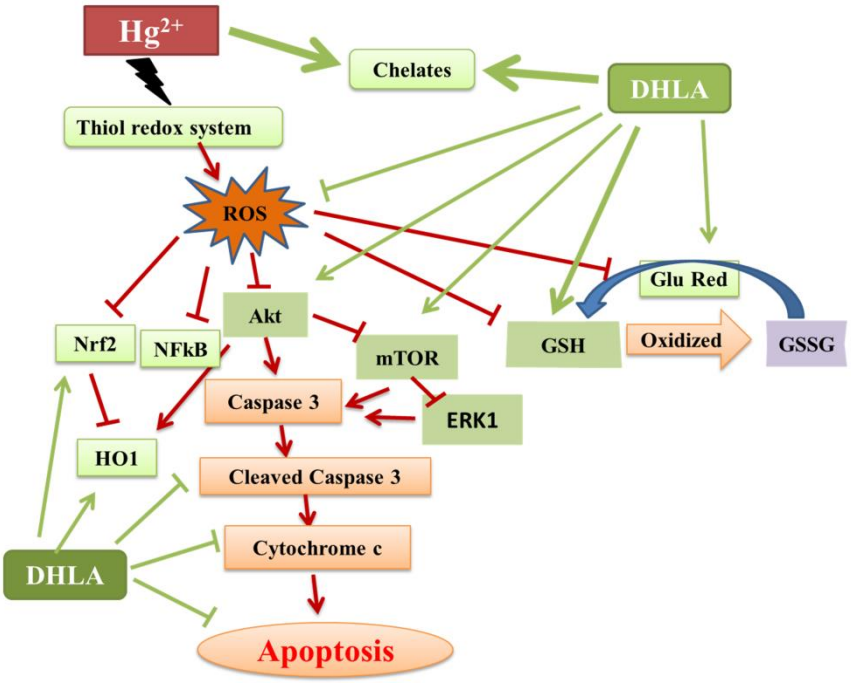

Figure 3.7: A simplified illustration of regulatory effects of DHLA against iHg-induced cytotoxicity in PC12 cells.

3.4. Discussions

All forms of Hg bind to sulfhydryl and selenohydryl groups, alter the tertiary and quaternary structure of proteins, and impair cell function (Bernhoft, 2011). Therefore, it can damage the function of any subcellular structure or organ. The major difference between organic and inorganic Hg is the mechanism of accumulation of these species in the cells. As a lipophilic substance organic Hg is more readily get bioaccumulated than that of inorganic form. Because of its lipophilic property organic Hg can cross blood brain barrier (BBB) more easily than iHg and induces neurological alterations (Ni et al., 2010). Although neuronal accumulation and alteration of iHg were also reported by Teixeira et al., (2018) and Aragão et al. (2018)), iHg get accumulated primarily in kidney and other organs, and cause detrimental toxicity in those organs (Orr et al., 2019). Even after getting accumulated, organic Hg can convert to iHg, and cause toxicity (Orr et al., 2019). Because of higher bioaccumulation property, the mechanisms of action of organic Hg have been studied extensively in molecular level, whereas mechanism of action in iHg-toxicity is still little explored, controversial and inconclusive, in spite of its increasing exposure to human. Little was known of the interaction and molecular mechanisms responsible for iHg-toxicity, especially in PC12 cells. Moreover, there was no study exploring the ameliorative effect of DHLA on iHg (Hg^{2+}) influenced cytotoxicity in PC12 cells. To explore the mechanisms of

iHg-mediated damage in PC12 cells and to verify the contribution of oxidative stress in iHg-cytotoxicity, in this research a set of indexes indicating cytotoxicity and oxidative stress were investigated in PC12 cells. In addition, the protective effects of DHLA as an antioxidant against Hg^{2+} induced toxicity were investigated. As a result, it reveals that Hg^{2+} induces cytotoxicity and cell death by apoptosis via intrinsic pathway in PC12 cells, through inhibiting mTOR and akt activation and increasing cytochrome c and active caspase 3. Again, Nrf2- HO1 pathway and inhibition of NFkB also contributed in Hg^{2+}-induced toxicity. On the contrary, DHLA pretreatment protected PC12 cells by inhibiting accumulation of iHg, upregulating mTOR, akt, GR, NFkB, Nrf2 and HO1.

Studies have shown that Hg in different forms can induce toxicity both in vivo and in vitro (Wang et al., 2001; Kim and Sharma, 2003; Chen et al., 2006; Wolf and Baynes, 2007; Zhou et al., 2009; Wang et al., 2017). Findings of this study indicated that Hg^{2+} induces cytotoxicity in PC12 cells (1.25 and 2.5 µM). Similar Hg-influenced toxicity in pheochromocytoma cells (PC12) (Parran et al., 2001), and lymphoma cells (EL4 and A20) (Kim and Sharma, 2003) have been reported.

LDH release into the culture media from the cells is an indication of cell-membrane damage. In this study, a significant LDH release indicating cell membrane damage was observed in Hg^{2+} (1.25 and 2.5 µM) exposed groups (Fig 3.2). Wolf and Baynes (2007) reported that Hg induced LDH release in bovine pulmonary artery endothelial cell monolayers. Kim and Sharma (2003) also reported that Hg increased the LDH level in lymphoma cells. In this study, a significant decline of intact DNA amount was detected in Hg^{2+}-treated PC12 cells. Similarly, Pieper et al. (2014) also reported Hg induced toxicity and DNA damage in human astrocytes. Kim and Sharma (2003) reported decrease in DNA synthesis upon Hg exposure.

Oxidative stress is a redox homeostasis imbalance, augmented by a combination of over produced free radicals and impaired antioxidants, and leads to disruption of energy metabolism, mitochondrial membrane potential dissipation, lipid peroxidation, and DNA impairment and proteins alteration. The enzymatic (superoxide dismutage, glutathione peroxidase (GPx) and catalase etc) and non-enzymatic (glutathione etc) endogenous or exogenous antioxidants can protect from these damages (Yang et al., 2016a). Reduced glutathione is the most abundant endogenous antioxidant, which acts in elimination of potential toxic metals and electrophiles, and thus protects against ROS. It presents abundant function in gene expression, membrane transport, DNA repair and apoptosis (Chatterjee, 2013). Several studies have stated that Hg^{2+} and $MeHg^{+}$, both have high affinity towards protective nucleophiles, for instance, –SH and –SeH (Aaseth et al., 2016). Consequently, they target essential antioxidants with thiol- and

selenol-molecules, for example, GSH and GPx; and thereby intensify oxidative stress (Bjørklund et al., 2019). Because of strong affinity for thiol groups, Hg can affect critical enzymes and structural components in the neuro cytoskeleton (Syversen and Kaur, 2012).

Upon Hg^{2+} exposure GSH and GR contents of the PC12 cells were reduced significantly, indicating a significant upsurge in ROS amount and oxidative stress. Increased ROS may cause the imbalance of oxidants and antioxidants by converting GSH to its oxidized form (GSSG). Perhaps reduced GR was unable to restore intracellular GSH by reducing GSSG and maintain the balance in reduced GSH. Similarly, Pal et al., (2012) reported GR decrease upon $HgCl_2$ exposure in hepatocytes. Wolf and Baynes (2007) found that high concentration of Hg ions caused GSH depletion and endothelial dysfunction in endothelial cell monolayers. In addition, ROS generation and oxidative stress induction by MeHg in rat cerebral cortex was reported by Yang et al., (2018a). Similarly, contribution of ROS in inorganic Hg-induced cytotoxicity was found by Kim and Sharma (2003) in murine T and B lymphoma cell lines. These studies provides evidence that Hg^{2+} has a cytotoxic effect in PC12 cells and involves ROS generation, GSH depletion, reduced GR, cellular membrane degradation, DNA damage and cell death.

To explore Hg^{2+}-mediated biomolecular mechanism/s in PC12 cells, protein expressions have been demonstrated in this research (Fig 3.6 and Fig 3.7). iHg exerts cytotoxicity and cell death *via* apoptosis induction in *in vivo* (Teixeira et al., 2018; Aragão et al., 2018) and *in vitro* (Liu et al., 2018) experiments. Aragão et al. (2018) reported similar oxidative stress induction, caspase 3 activity increase and apoptosis induction upon exposure to iHg in the motor cortex of Wistar rats. Similarly iHg induced oxidative stress and apoptosis was reported by Teixeira et al. (2018) in male Wistar rat. Hg induces cellular apoptosis by producing oxidative stress and activation of caspase cascade in human lymphocytes (Shenker et al., 2002). Inhibition of NFκB is recognized as a key to the apoptosis induction (Mathas et al., 2003). In this study, it was found that 2.5 μM of Hg^{2+} resulted in downregulation of NFκB and caspase 3, and upregulation of active caspase 3 and cytochrome c release, which indicated apoptosis induction in PC12 cells in the intrinsic pathway. Moreover, 2.5 μM of Hg^{2+} caused reduction of Nrf2 and HO1 in PC12 cells. Similarly, Ma et al., (2018) also reported $HgCl_2$ mediated oxidative stress and suppresstion of Nrf2-Keap1signalling in liver and kidney of laying hens. In summary, the results show that Hg^{2+} resulted in increasing oxidative stress revealed by GSH and downregulation of GR (Fig 3.2), DNA damage (Fig 3.2), cell membrane destruction and LDH leakage (Fig 3.1), downregulation of akt, mTOR, NFkB, Nrf2, HO1, ERK1 and caspase 3, and upregulation of active caspase 3 and cytochrome c release (Fig 3.4 and 3.5), and finally induces apoptosis (Fig 3.3 and 3.7) in

PC12 cells. In this study, Hg accumulation in the cells was also confirmed (Fig 3.6). Berntssen et al. (2003) also reported that Hg exposure causes significant Hg accumulation in Atlantic salmon.

α-LA and DHLA, both are well-recognized for their tremendous antioxidants and oxidative stress scavenging properties (Bjørklund et al., 2019). Both of them can react with heavy metals and other toxicants to support the primary antioxidant defense system in cellular injury. LAs are recommended for heavy metal detoxification, especially for strengthening Hg detoxifying process. DHLA is exclusive among other -SH containing biomolecules (e.g. glutathione and cysteine), where both of its thiol groups are reactive. The location of the two sulfur atoms in the ring structure of DHLA, creating an extraordinarily high electron density, provides special properties (for example, high reactivity in physiological conditions) that are not shared by other disulfides (Smith et al., 2004). Several studies have shown that LAs have protective effects against heavy metals (cadmium, aluminum, lead) or toxic drugs (gentamicin) induced oxidative stress (El-Maraghy et al., 2011; Sahin et al., 2017; Pande and Flora, 2002; Tahira et al., 2012). α-LA protects against MeHg-induced neurotoxicity through oxidative stress inhibition in rat cerebral cortex (Yang et al., 2015). Yang et al. (2016a) reported that α-LA reduces MeHg-mediated injury in rat cerebral cortex using antioxidant pathways. The ameliorative effects of α-LA against MeHg-influenced oxidative stress and intracellular Ca^{2+} dyshomeostasis was reported in cultured neurons of neonatal Wistar rats (Yang et al., 2016b).

In this study, the pretreatment of DHLA (50 µM) on Hg^{2+} (2.5 µM) toxicity in PC12 cells were examined. It was found that pretreatment of DHLA defends PC12 cells from Hg^{2+}-cytotoxicity through improving cell viability (Fig 3.1), elevating cell membrane integrity (Fig 3.1), decreasing DNA damage (Fig 3.2), ameliorating GSH and GR content (Fig 3.2), upregulating akt, p-akt, mTOR, NFκB, ERK-1, Nrf2 and HO1(Fig 3.4 and Fig 3.5), and downregulating active caspase 3 and cytochrome c release (Fig 3.5) and finally, limiting apoptosis (Fig 3.3 and Fig. 3.7). Moreover, this study confirmed DHLA-mediated alteration/inhibition of Hg accumulation (Fig 3.6), which indicates a strong possibility of complex formation between iHg and DHLA. Similarly, Gregus et al. (1992) proposed inorganic mercury excretion in the form of DHLA–Hg^{2+} complexes in *in vivo* study. Keith et al. (1997) also indicated removal of iHg in *in vivo* and *in vitro* model with LA by chelation. In addition, the complexation or chelate formation of α-LA and DHLA with Hg^{2+} was confirmed by Chekmeneva et al. (2010) with differential pulse voltammetric (DPV) method using the rotating Au-disk electrode. Main complexes were 1:1 Hg: DHLA, though 1: 2

complexes formation can be also inferred, and diverse Hg^{2+}-binding configurations were seen at different pH (Chekmeneva et al., 2010). Therefore, here it suggests that chelating properties of DHLA with Hg^{2+} might be strongly responsible with the inhibition of Hg accumulation and resulting protection of DHLA on iHg-induced toxicity in PC12 cells. Further study is required to confirm the complex formation and the molecular mechanisms behind this protection.

In this research, various antioxidant properties of DHLA (e.g. chelating metals, ROS scavenging, endogenous antioxidants regeneration and oxidative damage restoration) could be related to the protection. Yang et al. (2016a) reported that LA provided protection might be linked to free radical scavenging abilities and oxidative stress reduction. Besides, endogenous antioxidants (e.g. GSH) generation by LA is considered a major hallmark for Hg detoxifying (Patrick, 2002). It was found that pretreatment of DHLA on Hg^{2+} toxicity in PC12 cells upregulate akt, p-akt and mTOR, which are known to stimulate cell survival by improving antioxidant contents and hindering apoptosis. Similarly, Xie et al. (2012) showed that α-LA provided protection from ischemia-reperfusion injury via Akt/mTOR activation. In this research DHLA-mediated ERK1 upregulation, which is a pro-survival and anti-apoptotic protein was found. Similarly, Fontani et al. (2015) reported that α-LA down-regulates starvation-induced apoptosis in osteocytes by ERK1/2 signalling.

Again, it was found that pretreatment of DHLA upregulated Nrf2 and HO-1 protein expressions in Hg^{2+}-induced cytotoxicity. Similarly, Koriyama et al. (2013) reported Keap1/Nrf2-dependent HO-1induction-mediated α-LA-protection from oxidative stress in the RGC-5 cells. In this study, it was found that pretreatment of DHLA stimulated decrease of pro-apoptotic NFkB expression in Hg^{2+} treated PC12 cells. Similarly, Meng et al. (2008) reported that α-LA effectively mitigates high glucose-mediated apoptosis, promoting NFkB expression and decreasing caspase 3 and caspase 9 expressions in human umbilical vein endothelial cells. In this study it revealed that DHLA pretreatment attenuated the caspase 3 expression and total inhibition of cytochrome c release in Hg^{2+}-treated PC12 cells.

To summarize, these findings exhibit that DHLA impedes Hg^{2+} persuaded intrinsic apoptosis in PC12 cells via inhibiting Hg accumulation, improving GR content, inhibiting GSH oxidation, repairing DNA degradation, inhibiting LDH discharge and limiting cytochrome c leakage from mitochondria. The apoptosis inhibition by DHLA pretreatment upon iHg exposure was also further confirmed by the flow cytometry analysis. In addition, it revealed the possible involvement of Akt-mTOR and Nrf2-HO1 pathways, although further study is needed elucidating contribution of these pathways.

In conclusion, iHg has a cytotoxic effect in PC12 cells, even in a small concentration. Pre-treatment of DHLA attenuates Hg^{2+} induced oxidative damage and intrinsic apoptotic cell death. These findings give insight into the cellular alteration by iHg-toxicity and the complex mechanism involved antioxidant activities of DHLA on iHg-toxicity in PC12 cells, as well as in other cells. These outcomes may propose that DHLA will be as a dietary supplement to combat iHg-toxicity in human and animals. In addition, DHLA will be possibly metal based new chemical therapeutics in cancer/tumor treatments.

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Chapter 4: Selenium protection against inorganic mercury induced cytotoxicity *via* modulating oxidative stress and intrinsic apoptosis in PC12 cells

Abstract

Mercury (Hg) in its all forms, including inorganic Hg (iHg) is an environmental contaminant due to toxicity and diseases in human. However, a little has been known about the underlying mechanisms responsible for iHg toxicity. Selenium (Se) is an essential trace element, and recognized as an antioxidant and protective agent against metal toxicities. The purpose of this research was to elucidate the molecular mechanism of iHg induced toxicity as well as, to investigate ameliorative effects of Se against iHg-induced toxicity in PC12 cells. Cytotoxic assays have been shown that iHg (5 μM) induced oxidative stress and intrinsic apoptotic cell death *via* oxidizing glutathione, damaging DNA, degrading cell membrane integrity, down-regulating mTOR, p-mTOR, akt, ERK1 and caspase 3, and up-regulating cleaved caspase 3 and cytochrome c release in PC12 cells 48 h after incubation. Again, co-treatment of Se (5 μM) appeared to inhibit intrinsic apoptosis and oxidative stress induced by iHg (5 μM) *via* boosting GPx contents, improving reduced form glutathione contents, limiting DNA degradation, improving cell membrane integrity, up-regulating mTOR, p-mTOR, akt, ERK1 and caspase 3, and down-regulating cleaved caspase 3 and cytochrome c leakage in PC12 cells. In addition, flow cytrometry analysis confirmed that apoptosis induced by iHg toxicity was improved by Se-co-exposure. The contribution of nrf2 and HO-1 activation was not significant in iHg-toxicity or Se-protection mechanisms in PC12 cells. In conclusion, these results suggested that glutathione level decrease plays a critical role in iHg induced oxidative stress and co-treatment of Se attenuates iHg-cytotoxicity through its antioxidant properties.

4.1. Introduction

Mercury (Hg) is classified as a hazardous heavy metal among the most toxic metals existing in environment and causes human intoxication. It is labeled as the third most dangerous substance by Agency for Toxic Substances and Disease Registry (ATSDR). Hg has three forms in environment: elemental Hg (Hg^0), inorganic Hg (Hg^+ and Hg^{2+}), and organic Hg, such as methylmercury (CH_3Hg, MeHg) and ethylmercury (C_2H_5Hg, EtHg) (Carocci et al., 2014). Sources of organic Hg include seafood and medical products. Hg^0 is easily absorbed via inhalation while burning fossil fuels, cement production, incineration of solid wastes and

leakage from thermometers, barometers, and batteries. Inorganic Hg (iHg) is a component of dental amalgams, medications, germicidal soaps and skin creams. Hg can cross blood brain barrier (BBB), thereupon, it elicits numerous cytotoxic effects and causes deleterious consequence on central nervous system. Hg has been reported to cause neurological disorder, behavioral disorder, memory loss, Alzheimer, motor dysfunction, decreased fertility, development problem in fetus, abnormal offspring, autism, immunity impairment, sensory disabilities, disturbances in listening and vision, increased fatigue, renal and cardiovascular dysfunction (EPA 2017; Rahman et al., 2019). Recent studied have shown that Hg persuades toxicity in different cell lines including PC12 cells via generation of reactive oxygen species (ROS) and oxidative damage (Parran et al., 2001; Chen et al., 2010; Chan et al., 2017).

The essential micronutrient, selenium (Se), has gained attention for its antioxidant properties. Human usually takes Se through the diet. Predominantly high Se-containing food sources are spinach, Brazil nuts, meats, crab, shellfish, tuna fish, mushrooms, oysters, seeds, mussels, and eggs (Hossain et al., 2018). Se is highly bioavailable in its organic forms, for instance, selenomethionine and gamma-glutamyl-Se-methylselenocysteine. Additionally, inorganic Se compounds such as sodium selenite are also available. Se uptake is mandatory for the appropriate functioning of immune system, thyroid metabolism, disease progression, reproduction, and reducing cancer risk (Rayman, 2012). As a component of selenoproteins, it has important structural and enzymatic roles in human body. One of the prime selenoprotein, glutathione peroxidase (GPx) is recognized to catalyze reduction of peroxide compositions and phospholipid peroxides using glutathione (GSH), and deliver numerous antioxidant protections (Reeves et al., 2009).

PC12 cell line was used in this research to determine the potential protective roles of Se against iHg-induced cytotoxicity. PC12 cells have been used as a model of neural cells (Takeda et al., 2000; Zhang et al., 2010). Protecting effects of antioxidants with toxic metals upon co-exposure have been reported recently. For example, Se has been reported to reduce cadmium-induced cytotoxicity in PC12 cells by reducing oxidative stress and inhibiting apoptosis (Hossain et al., 2018); ameliorative effects of Se upon arsenic toxicity have been reported in PC12 cells by positively regulating autophagy/apoptosis (Rahman et al., 2018); while cytoprotective effects of zinc on cadmium toxicity in PC12 cells have been reported (Rahman et al., 2017). In addition, protection of Se against Hg-induced toxicity has been studied *in vivo* (Kehrig et al., 2016; Sakamoto et al., 2013; Wang and Wang, 2017; Orr et al., 2019) and *in vitro* (Wang et al., 2001; Frisk et al., 2003; Wang et al., 2017; Meinerz et al.,

2017). Though, the exact biomolecular mechanism/s of cytoprotective effects of Se against Hg-induced toxicity remain/s ambiguous. Additionally, the antioxidant effects of Se on iHg-induced toxicity in PC12 cells have not been studied yet. Thus, in this study, it was hypothesized that Se has inhibitory effects against iHg-toxicity and provides protection against oxidative stress in PC12 cells. Therefore, for the first time, to investigate this hypothesis several cytotoxicity investigation techniques were used after co-exposure of Se and/or iHg in PC12 cells for a specified period of time.

4.2. Methodology

4.2.1. Cell culture

PC12 cells were used in this study like as previous studies (Hossain et al., 2017; Rahman et al., 2017, 2018). Cells were cultured in a humidified incubator with 5% CO_2 at 37°C. The cells were cultured in 25-cm^2 flasks for 48 h in DMEM (Sigma-Aldrich, USA) supplemented with 10% FBS (Biosera, USA). After that, the medium was replaced with serum/serum-free DMEM with or without various concentrations of $HgCl_2$ (Hg^{2+}) (0-5 μM) as a source of iHg and Na_2SeO_3 (SeO_3^{2-}) (0-10 μM) as a source of Se, separately. Then the final combinations were decided, and the chosen exposure conditions for iHg and Se both were 5 μM for 48 h at 37°C.

4.2.2. Cell viability

Trypan blue (Bio-Rad, USA) exclusion assay was used to analyze cell viability of PC12 cells exposure to iHg and/ Se. Cells were cultured and preincubated for 48 h in DMEM supplemented with 10% FBS. Then, the cells were treated with/without Se (5 μM), iHg (5 μM) or Se and iHg (5 μM+5 μM) together. After 48 h of incubation, total cells and trypan blue-staining cells were counted as described by Hossain et al. (2017). To confirm biological reproducibility, each experiment was performed at least six times.

4.2.3. Lactate dehydrogenase (LDH) activity assay

PC12 cells were co-treated with/without Se (5 μM), iHg (5 μM) or Se and iHg (5 μM+5 μM) together. LDH activity assay of the medium was performed using a non-radioactive cytotoxicity assay kit (Promega; Fitchburg, WI, USA) as described by Hossain et al. (2018) to examine cell-wall integrity. LDH activity was expressed as LDH activity/1 × 10^6 cells. These experiments were done at least four times to ensure reproducibility.

4.2.4. Genomic DNA isolation from cells

PC12 cells were cultured and treated with/without Se (5 μM), iHg (5 μM) or Se and iHg (5 μM+5 μM) together for 48 h. Then the cells were harvested, and washed with ice cold 1 × PBS. The genomic DNA was extracted with high pure PCR template preparation kit (Roche Diagnostics, Germany) as described by Rahman et al. (2018).

4.2.5. Agarose gel electrophoresis of genomic DNA

The intact DNA obtained was observed using agarose gel electrophoresis. The same amounts of isolated DNA from the cells treated with Se (5 μM), iHg (5 μM) or Se and iHg (5 μM+5 μM) together for 48 h, were ran in electrophoresis on a 1.5% agarose gel as explained by Hossain et al. (2018). To secure reproducibility this experiment was performed at least four times.

4.2.6. Measurement of intracellular free sulfhydryl (SH) levels

Intracellular free SH levels were assessed using the method described by Rahman et al. (2017). Cells were pre-incubated for 48 h and exposed to Se (5 μM), iHg (5 μM) or Se and iHg (5 μM+5 μM) together, harvested, washed with 1 × PBS, and cellular free sulfhydryl (SH) levels were analyzed following the method described in previous research (Hossain et al., 2018). The experiments were done three times for reproducibility.

4.2.7. Flow cytometry analysis for apoptosis detection

Cell death process of PC12 cells was evaluated using flow cytometry by Annxin V-FITC apoptosis detection kit (BioVision, USA). PC12 cells were treated with/without Se (5 μM), iHg (5 μM) or Se and iHg (5 μM+5 μM) together for 48 h; and harvested. As described in Hossain et al., 2020, the flow cytometry was performed. Cells were then analyzed using BD FACSVerse^{TM} flowcytometer (BD Biosciences). The experiments were done triplicate to ensure reproducibility.

4.2.8. Western blot analysis for determination of protein expression

PC12 cells were cultured, exposed with/without Se (5 μM), iHg (5 μM) or Se and iHg (5 μM+5 μM) together and harvested after 48 h incubation. Western blot analysis was performed according to Hossain et al. (2018). In the western blotting assays, following antibodies were used; Polyclonal antibodies against β-actin (cat# 4967, Cell Signalling

Technology), glutathione peroxidase-1 (AB22604, Abcam), mTOR (cat# 2972, Cell Signalling Technology), phosphorylated mTOR (SC-293133, Cosmo Bio Co., LTD), akt (cat# 4691, Cell Signalling Technology), p-akt (9267, Cell Signalling Technology), Nrf2 (PM069, Medical and Biological Laboratories Co., Japan), Heme oxygenase 1 (GTX101147, GeneTex), ERK1 (610031, BD Transduction laboratories), caspase-3 (GTX110543, GeneTEX), cleaved caspase-3 (cat# 9661, Cell Signalling Technology) and cytochrome c release Apoptosis Kit (Q1A87-1KIT, CalbiochemR). Each experiment was performed at least four times.

4.2.9. Statistics

Data are stated as the mean ± standard error of mean (SEM). Single-factor analysis of variance (ANOVA) followed by unpaired Student's *t*-test were executed for statistical analyses. Either $p<0.05$ or $p<0.01$ difference was considered as statistically significant.

4.3. Results

4.3.1. Cell Viability

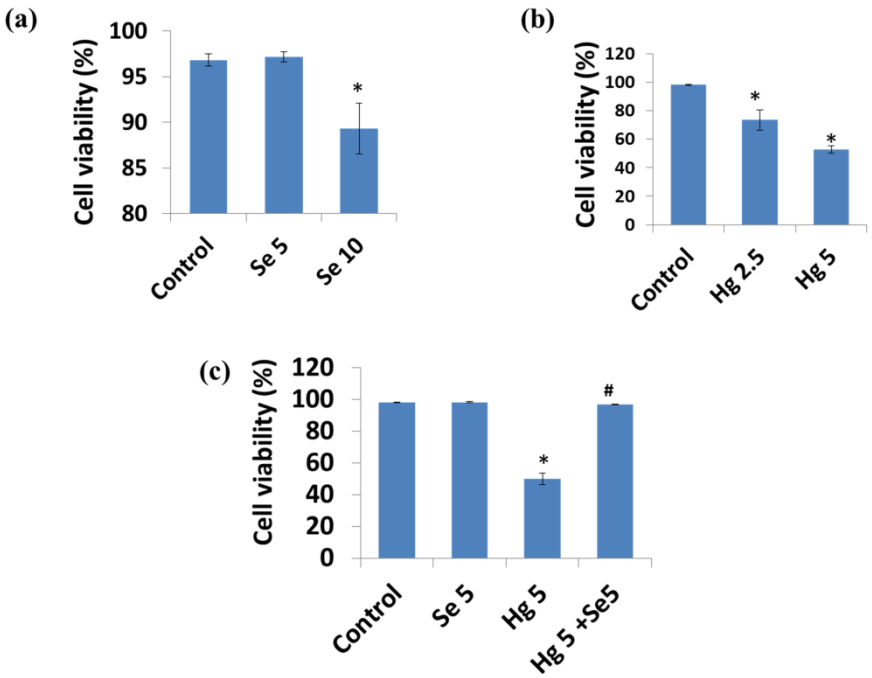

Figure 4.1: (a) Cell viability of PC12 cells cultured in medium with 0-10 µM of Se, (b) Cell viability of PC12 cells cultured in medium with 0-5 µM of iHg, (c) Cell viability of PC12 cells after co-exposure with Se and iHg (5 µM+5 µM) for 48 h incubation. Error bars indicate

mean ± SEM (n= at least 6). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group

Cell viability analysis using trypan blue straining was used to determine the effect of Se on Hg toxicity. Fig. 4.1A demonstrates the cell viability of cultured PC12 cells with different concentrations of Se after 48 h of incubation. The viability of PC12 cells was not declined till 5 μM of Se exposure to the cells. The results suggested that 5 μM of Se was not toxic for PC12 cells and it was decided that 5 μM of Se will be employed for further studies. Likewise, toxic Hg concentration was chosen from cell viability of PC12 cells treated with 0, 2.5 and 5 μM iHg (Fig. 4.1B) and finally 5 μM of iHg concentration was selected for combined experiment.

The cell viability of the simultaneous exposure of iHg and Se was determined. Fig. 4.1C showed cell viability of PC12 cells treated with iHg (5 μM), Se (5 μM) and both metals (iHg + Se, 5 μM+5 μM) for 48 h. It was found that the cell viability was significantly declined ($p<0.05$) when the cells were treated with iHg (5 μM) compared to the cells without treatment of metal ion. Conversely, the cells treated with only 5 μM of Se did not show any significant difference in cell viability when compared to that of the control cells. The cells exposed together with iHg (5 μM) and Se (5 μM) showed significant increase ($p<0.05$) of cell viability than the cells exposed to iHg (5 μM) alone. These findings suggested that Se has a protective effect on iHg-induced toxicity in PC12 cells.

4.3.2. Cell Membrane Damage

To investigate the cell membrane integrity two methods were employed. Fig. 4.2A shows the cell membrane damage of PC12 cells treated with iHg (5 μM), Se (5 μM) or combined exposure of iHg and Se (5 μM+5 μM) for 48 h of incubation. Here, green color represents intact cell membrane, and red color represents ruptured cell membrane. As shown in the Fig. 4.2A, compared to the cells of control cells the red colored cells were significantly ($p<0.05$) increased when cells were treated with iHg (5 μM) alone. On the other hand, when cells were co-exposed with iHg and Se, green colored cells which meant intact cell membrane were increased significantly ($p<0.05$). These results suggested that Se has a protective effect on iHg-induced toxicity and cell membrane damage in PC12 cells.

For verification of these results, another method, LDH assay was employed to examine cell membrane damage. Fig. 4.2B demonstrates the LDH release from cells to the culture media which was used for incubation of PC12 cells with iHg (5 μM), Se (5 μM) or combined

exposure of iHg and Se (5 µM+5 µM) for 48 h. The LDH activity in the culture media of the cells treated with only iHg (5 µM) showed a significant ($p<0.05$) rise (almost six fold) compared to that of control group media. Again, the LDH activity of the culture media of the cells co-treated with iHg (5 µM) and Se (5 µM) was significantly ($p<0.05$) reduced compared to the LDH level of the cells treated with iHg (5 µM) alone. The results together with Fig. 2A result confirmed that Se reversed the cell membrane damage caused by iHg and protected the cells from iHg toxicity.

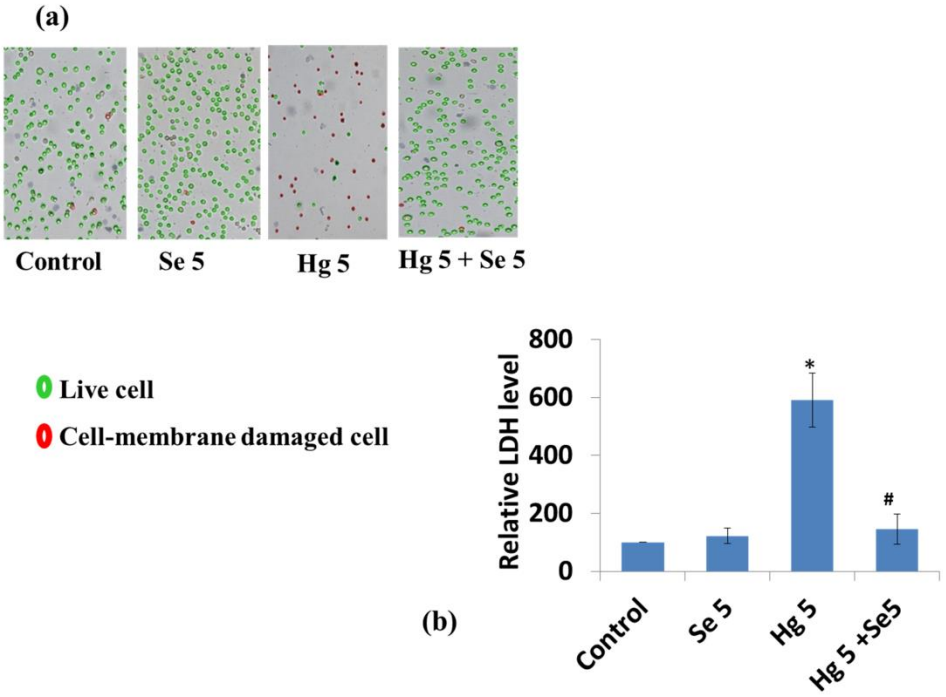

Figure 4.2: (a) Detection of cell wall damage in the cultured cells using trypan blue staining when cells were treated with/without Se (5 µM), iHg (5 µM) or Se and iHg (5 µM+5 µM) together for 48 h. (b) LDH levels in the cultured medium for PC12 cells treated with/without Se (5 µM), iHg (5 µM) or Se and iHg (5 µM+5 µM) together for 48 h. Each column is shown as mean ± SEM, n=3. * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group

4.3.3. DNA Damage

To evaluate DNA damage in PC12 cells Agarose gel electrophoresis method was applied. Fig. 4.3A shows the agarose gel electrophoresis of the cells exposed to iHg (5 µM), Se (5 µM) or concurrently exposed to iHg and Se (5 µM+5 µM) after 48 h of incubation, whereas Fig. 4.3B shows quantification of Fig 4.3A. The intact DNA amount extracted from the cells treated

with iHg (5 μM) alone was significantly ($p<0.05$) deteriorated when compared to the cells of control group. On the contrary, the amount of intact DNA extracted from the cells concurrently exposed to both iHg (5 μM) and Se (5 μM) was significantly ($p<0.05$) improved than the cells exposed to iHg (5 μM) alone. This result indicates that Se inhibits iHg-induced DNA damage.

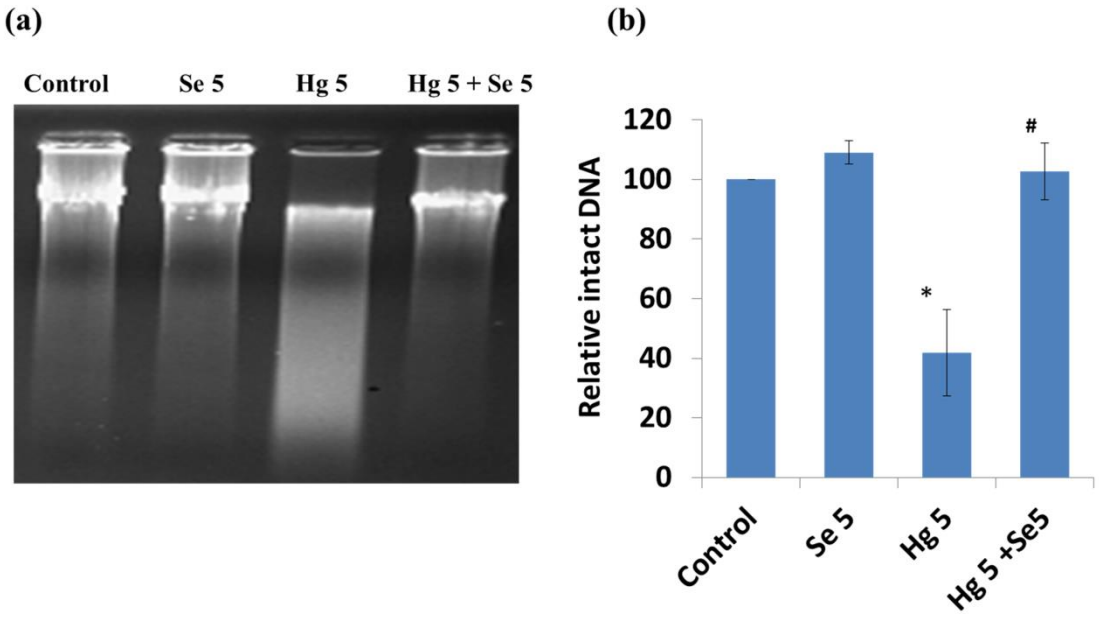

Figure 4.3: (a) Agarose gel electrophoresis of DNA isolated from PC12 cells treated with/without Se (5 μM), iHg (5 μM) or Se and iHg (5 μM+5 μM) together for 48 h. Intensity of band obtained from DNA in the cells treated was shown below the electrophoresis band (b). Error bars are mean ± SEM (n=4). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group

4.3.4. Contents of reduced glutathione

Adequate amount of reduced GSH, which oxidizes damaging ROS, is crucial for maintaining homeostasis of the cells. Decrease in reduced GSH amount is an indirect marker of ROS increase in the cells. In this study, GSH amount was measured to investigate ROS production in cells. Fig. 4.4A shows the GSH content of the cells treated with iHg (5 μM), Se (5 μM) or both metals (5 μM+5 μM) after 48 h of incubation. The relative content of GSH was significantly ($p<0.05$) deduced in cells treated with only iHg compared to that of control group. Contrarily, when cells were co-treated with iHg and Se (5 μM+5 μM), cellular GSH content was significantly ($p<0.05$) surged, compared to only iHg (5 μM) treated group. These

outcomes suggested that Se can protect PC12 cells from iHg induced GSH decrease and ROS increase.

In addition, GPx is an enzyme, whose major role is to protect organisms from oxidative damage by catalyzing the reduction of peroxides, was assessed by western blotting. Fig. 4.4B shows the GPx contents of the cells exposed to iHg (5 μM), Se (5 μM) and both metals (5 μM+5 μM) after 48 h of incubation. The GPx content was significantly ($p<0.05$) increased when cells were treated with only Se (5 μM), compared to the control group. Again, the GPx content was significantly ($p<0.05$) increased when cells were treated with both metals (5 μM+5 μM) compared to the cells treated with iHg (5 μM) alone. From the results, Se protects PC12 cells from oxidative damages caused by iHg *via* elevating GSH and improving GPx content.

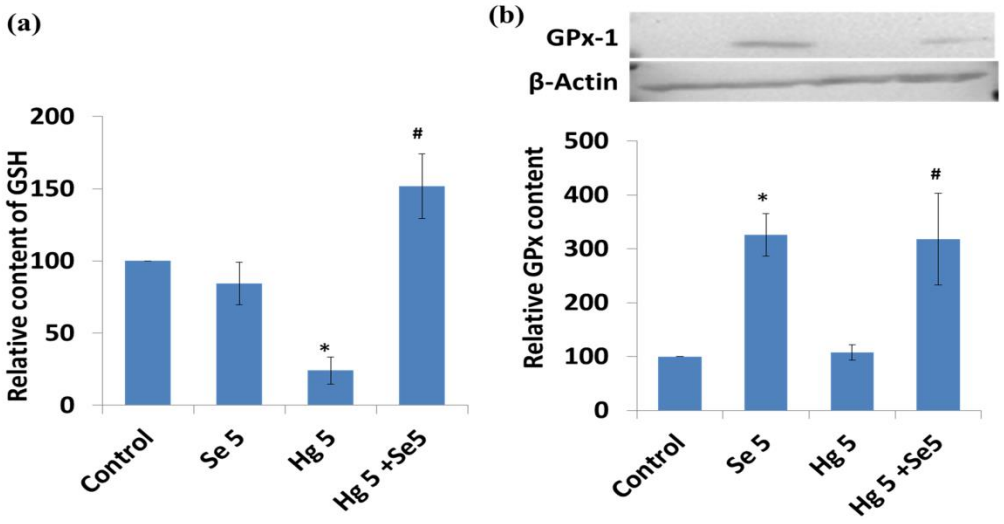

Figure 4.4: (a) GSH contents and (b) glutathione peroxidase (GPx) contents in the cultured cells treated with/without Se (5 μM), iHg (5 μM) or Se and iHg (5 μM+5 μM) together for 48 h. Error bars are mean ± SEM (n=3 and 5 respectively). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group

4.3.5. Apoptosis detection

Flow cytometry analysis was employed to detect Hg-induced apoptosis and Se-induced protection on Hg-induced apoptosis in PC12 cells. Fig. 4.5 demonstrates the percentage of apoptosis in cells treated with iHg (5 μM), Se (5 μM) or both metals (5 μM+5 μM) after 48 h of incubation. It was found that the percentage of apoptotic cell was significantly ($p<0.05$)

increased in the cells treated with only iHg (5 µM), compared to the cells treated with no treatment. Again, the percentage of the apoptotic cells were significantly ($p<0.05$) depleted when cells were treated with iHg (5 µM) and Se (5 µM) together, than the cells treated with iHg (5 µM) alone. These results suggested that iHg induces apoptosis and Se can inhibit iHg-induced apoptosis in PC12 cells.

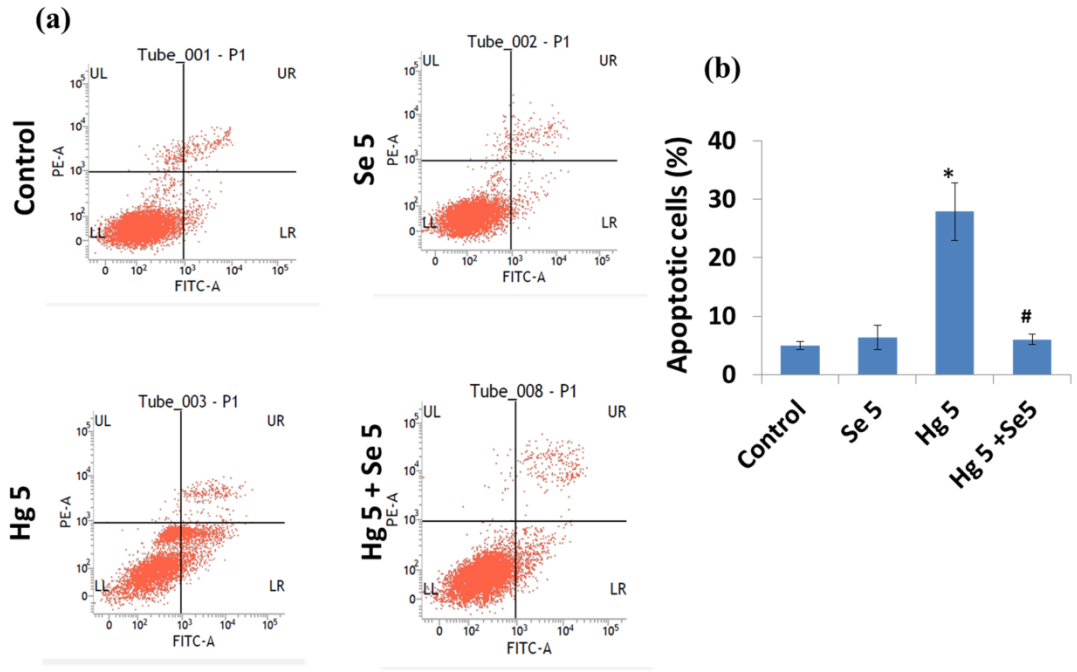

Figure 4.5: (a) Flow cytometry analysis of cultured PC12 cells, strained with Annexin V-FITC/PI after treatment with/without Se (5 µM), iHg (5 µM) or Se and iHg (5 µM+5 µM) together for 48 h and (b) percentage of apoptotic cells. Error bars are mean ± SEM (n=3). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group.

4.3.6. Regulation of protein expressions

To investigate the molecular mechanism/s responsible for Se-protection against Hg-induced toxicity and apoptosis, western blotting analyses were done. Fig. 4.6 shows the protein expressions and quantification of the cells exposed to iHg (5 µM), Se (5 µM) or both metals (5 µM+5 µM) for 48 h of incubation.

The protein expression of mTOR (mammalian target of rapamycin), which regulates protein synthesis, cellular growth and proliferation in response to growth factors, nutrients, and stress; and its phosphorylated form p-mTOR were analyzed. The protein level of mTOR and p-mTOR, both were significantly ($p<0.05$) decreased in cells treated with only iHg (5 µM)

when compared to the control group cells. Interestingly, both protein expressions were significantly ($p<0.05$) increased in cells treated with iHg (5 μM) and Se (5 μM) together, compared to the cells treated with only iHg (5 μM). Another key controller akt, which regulates cellular metabolism, survival, apoptosis, transcription, cell-cycle progression and proliferation; and its phosphorylated form p-akt were investigated. akt protein expression was significantly ($p<0.05$) declined when cells were exposed to iHg (5 μM) alone, compared to the control cells. Again, simultaneous exposure of iHg and Se (5 μM+5 μM) in PC12 cells showed a significant ($p<0.05$) increase in akt expression compared to the group treated only with iHg (5 μM). There was a significant ($p<0.05$) decrease in p-akt level upon iHg (5 μM) exposure to the PC12 cells compared to the control group, thought the level of p-akt was not changed upon simultaneous exposure of iHg and Se (5 μM+5 μM) compared to only iHg (5 μM) exposed cells. These findings suggested mTOR and akt activation influenced in Se induced protection in iHg-toxicity in PC12 cells.

ERK1, a member of MAPK cascades and activated by extracellular signals, plays a vital role in regulation of cell growth, differentiation and inhibition of apoptosis. As shown in Fig. 4.6, the protein expression of ERK1 was significantly ($p<0.05$) down-reglated in cells treated with iHg (5 μM) alone when compared to the control group. However, the ERK1 expression was significantly ($p<0.05$) improved in cells treated with iHg and Se (5 μM+5 μM) together, compared to the cells of iHg (5 μM) alone treated group. This result indicated that ERK1 increased by Se inhibited Hg-induced apoptosis.

The activation of Nrf2 pathway plays a critical role in inhibition of inflammation and Nrf2-mediated antioxidant gene expression can reduce ROS production. One of the Nrf2-mediated antioxidant is HO-1. Involvement of NRF2-HO1 pathway was examined in Se-protection in Hg-toxicity in PC12 cells; however, Fig. 4.6 showed that there was no significant change of Nrf2 or HO1 protein levels in PC12 cells upon different exposures.

To investigate Hg-induced cell-death mechanism, apoptotic proteins caspase 3, cleaved caspase 3 and cytochrome c released were analyzed (Fig. 4.6). In the only-iHg (5 μM) treated cells showed a significant ($p<0.05$) decrease in caspase 3 and increase in cleaved caspase 3 compared to the control group cells. Again, the cells treated with simultaneous exposure of iHg and Se (5 μM+5 μM) showed a significant ($p<0.05$) increase in caspase 3 and decrease in cleaved caspase 3 compared to the cells treated with only iHg (5 μM). Similarly, cytochrome c released into cytosol, which is crucial for mitochondrial membrane potential loss and initiation of intrinsic apoptosis, was significantly ($p<0.05$) increased in cells treated with iHg

(5 µM) alone, compared to the control group. However, cytochrome c released was significantly ($p<0.05$) decreased in cells treated with iHg and Se (5 µM+5 µM) together, compared with cells treated with iHg (5 µM) alone. From these results, it was confirmed that iHg is able to induce intrinsic apoptosis, and Se is capable of inhibiting iHg-induced intrinsic apoptosis in PC12 cells.

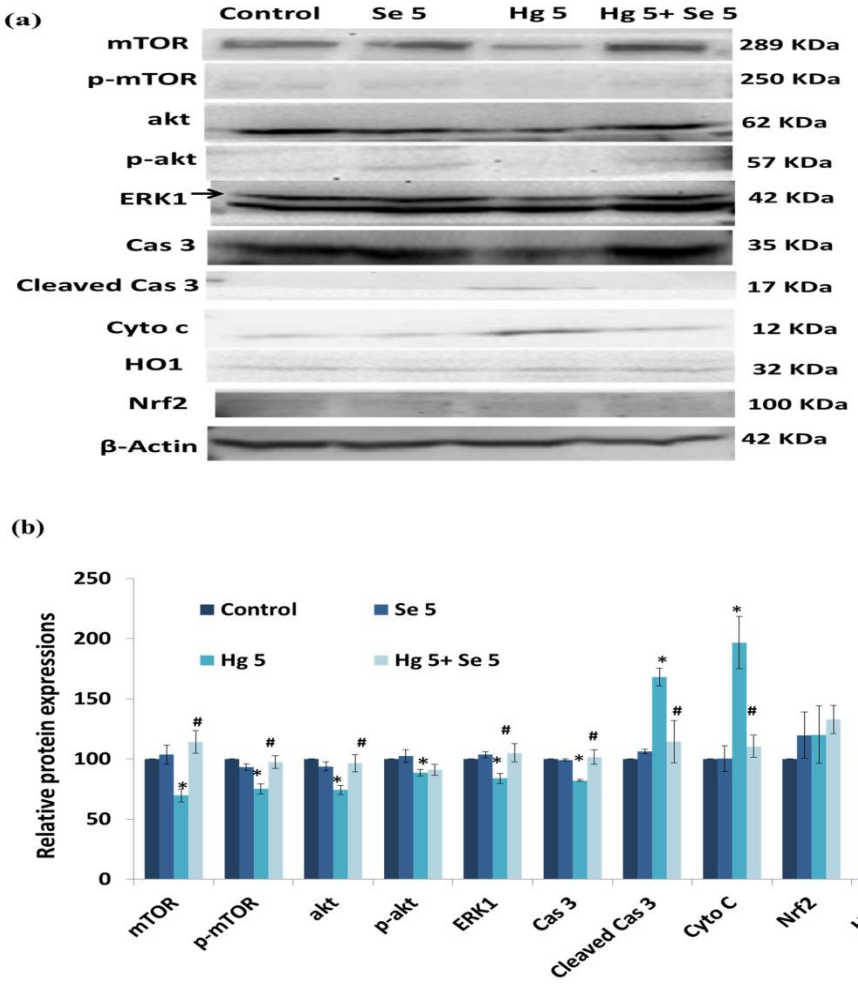

Figure 4.6: (a) Relative protein expression and (b) quantification of mTOR, p-mTOR, akt, p-akt, ERK1, caspase 3, cleaved caspase 3, cytochrome c, Nrf2, HO-1 and β-actin in the cultured cells treated with/without Se (5 µM), iHg (5 µM) or Se and iHg (5 µM+5 µM) together for 48 h. Error bars are mean ± SEM (n= atleast 4). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group.

However, co-treatment of Se (5 µM) showed significant hindrance against iHg (5 µM) on the activation of apoptotic proteins. These results suggested that Se (5 µM) co-treatment

obstructed iHg (5 µM)-induced intrinsic apoptosis in PC12 cells. Fig. 4.7 showed a schematic diagram of Se protection on iHg-induced cytotoxicity in PC12 cells.

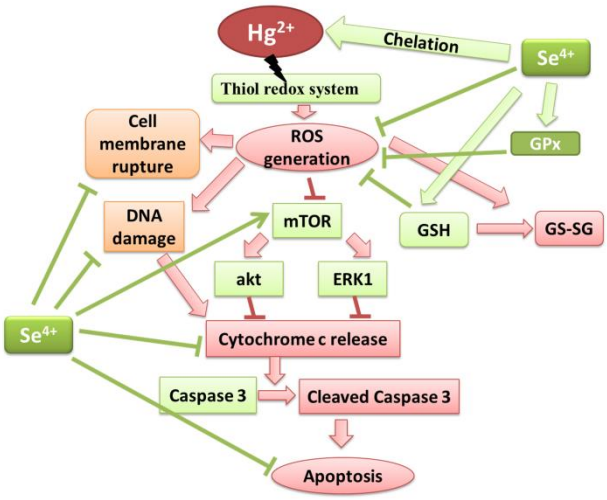

Figure 4.7: Schematic diagram of Se-protection on iHg-induced cytotoxicity in PC12 cells.

4.4. Discussion

One of essential elements, Se plays a vigorous role in antagonizing toxicity exerted by iHg. In this study, it was hypothesized that Se has alleviative on iHg-induced toxicity. The protective effects of Se on iHg ($HgCl_2$)-induced toxicity using different cytotoxicity assays in PC12 cells ware investigated. It was evident that Se has an ameliorative effect on iHg-induced toxicity. It was found that Se can inhibit iHg-induced intrinsic apoptosis in PC12 cells *via* inhibiting LDH release (Fig. 4.2B), increasing cellular GSH and GPx1 (Fig. 4.4), decreasing DNA damage (Fig. 4.5), upregulating akt, mTOR, and ERK1; and downregulating cleaved caspase 3 and cytochrome c (Fig. 4.6)

Previous studies revealed that Hg-induces toxicity, both *in vivo* and *in vitro* (Wang et al., 2001; Yin et al., 2007; Wang et al., 2009; Zhou et al., 2009; Fujimura and Usuki, 2015; Wang et al., 2017; Hossain et al., 2020). These reports have already proven that Hg compromised cytosolic thiol system, decreased mitochondrial membrane potential, increased ROS generation, consequent induction of an oxidative burst and lipid peroxidation. Similarly, in this study, it was found that compared with the control group, PC12 cells incubated with iHg (5 µM) revealed significant cell viability loss (Fig. 4.1), cell membrane integrity loss (Fig. 4.2A), LDH release (Fig. 4.2B), DNA damage (Fig. 4.3), GSH depletion (Fig. 4.4), and cell death in apoptosis process (Fig. 4.5).

The mechanisms of iHg-induced toxicity might be related to oxidative stress induced by either deficiency of oxidative defense capacity and/or excessive-formation of ROS. Several

line of studies reported that Hg ion has a strong affinity towards thiol (-SH) and selenol (-SeH) molecules, which have antioxidant properties, such as GSH and GPx. Thus, by weakening oxidative defense capacity iHg can be concluded to increase oxidative stress and augment ROS. Over-production of ROS disrupts energy metabolism, impairs various mitochondrial enzyme's actions those play key roles in defending oxidative damage (Battin and Brumaghim, 2009). To confirm the effects of iHg in ROS generation, GSH was monitored in PC12 cells. It was demonstrated here that treatment of iHg induced decreases of GSH, which is one of the highly abundant cellular scavengers of ROS. It was also found a significant upsurge in LDH release and DNA damage upon iHg exposure. Similarly, a significant reduction of GSH has been reported by iHg exposure in glioma cells (Lee et al., 2001). Wolf and Baynes (2007) reported Hg induced LDH release in bovine pulmonary artery endothelial cells. Pieper et al. (2014) confirmed that Hg species induced DNA damage in cultured human astrocytes. My results suggest that iHg induced toxicity is mediated by modulation of ROS and causes in increasing membrane permeability and DNA fragmentation. To elucidate the molecular mechanism of iHg induced toxicity in PC12 cells, several protein expressions were explored, where 5 μM of iHg inhibited pro-survival mTOR, akt and ERK1 protein expressions. My results also demonstrated that iHg induced upregulation of cleaved caspase 3 and cytochrome c in the cytosol, which are pro-apoptotic proteins. In addition, flow cytometry analysis proved iHg induced apoptosis in PC12 cells. In addition, involvement of Nrf2 and HO1 protein expressions were also examined, though were found insignificant. These results together with cellular toxicity parameters, propose that iHg induced toxicity in PC12 cells modulates intrinsic apoptosis cell death involving akt-mTOR pathway.

Se contributes regulatory roles in cell growth and survival, and cytotoxicity. Se is an essential trace element and well known for its antioxidant properties, is taken up by human predominantly from grains, cereals and meat. A complex reaction converts inorganic Se compounds (such as selenate and selenite) to their organic forms and vice versa, which can be enzymatically catalyzed (Zeng, 2009). Se with organic forms is better accumulated and retained in body rather than inorganic forms. Organic forms of Se in foods remain in reduced states (Se (−2)), whereas inorganic forms contain oxidized states (Se (+4) and Se (+6)); though after absorption all forms of Se are reduced to Se (−2) form using GSH and NADPH (Hossain et al., 2018). The mechanisms of Se for ameliorative effects on metal toxicities in cellular system are as follows-inhibition of metal absorption, or detoxification by complex formation with metals, or through antioxidant effects by increasing

seleno-proteins (e.g., GPx) activity, or maintaining homeostasis in the redox state by increasing GSH amount, or combinations of them. Protective effect of Se on lead-induced toxicity in chicken testes (Wang et al., 2017) and cadmium-induced toxicity in PC12 cells (Hossain et al., 2018) have been reported. El-shenawy and Hassan (2008) reported the ameliorative effect of Se on kidney damage induced by $HgCl_2$ in rats. Further study will be needed to confirm the inhibitory effect of Se in Hg induced toxicity in both cellular and molecular level.

Previous documents have shown that iHg has an affinity to bind with Se, and Se could prevent oxidative stress generated by iHg compounds. However, there are only few studies to explore the ameliorative effect of Se on iHg under *in vitro* conditions. Present study showed a couple of key roles of Se (5 μM) protection on iHg (5 μM)-induced cytotoxicity in PC12 cells in simultaneous exposure. In this study, It was found that Se inhibited iHg induced cytotoxicity in PC12 cells by raising cell survival (Fig. 4.1); enhancing membrane integrity (Fig. 4.2); limiting DNA damage (Fig. 4.3); improving cellular GSH amount (Fig. 4.4A); boosting GPx-1 protein expression (Fig. 4.4B); increases of mTOR, p-mTOR, akt and ERK1 (Fig. 4.6); and diminishing the pro-apoptotic protein expressions of cleaved caspase 3 and cytochrome c (Fig. 4.6); and finally arresting apoptosis (Fig. 4.5). These protection provided by Se is estimated to be resulted from the antioxidant properties of Se.

To investigate in detail about the antioxidant properties of Se on iHg toxicity, GPx-1 (one of the important sub-types of glutathione peroxidase) was scrutinized. It was found that upon exposure of Se to cells, the GPx-1 expression was enhanced indicating an effective defense against iHg-toxicity. Similarly, Schnabel et al. (2008) reported that Se improved GPx-1 content in coronary artery endothelial cells *in vivo* and *in vitro*. GPx is an enzyme that catalyzes the reduction of peroxides (H_2O_2 or lipid peroxides) by using GSH as a cofactor, by the enzymatic activity of GPx. In this study, it was found that Se induced increase of GSH in iHg-toxicity. The GSH level was significantly increased in cells exposed to both Se and iHg, compared to the cells exposed to only iHg. Se induced GSH increase in PC12 cells was reported in arsenic toxicity (Rahman et al., 2018). Increased GSH may modulate DNA-repair activity (Chatterjee, 2013). It was also found that Se induced protection from DNA damage induced by iHg toxicity. Similarly, Baliga et al. (2006) reported that Se and Se induced GPx-1 protected mammalian cells against DNA damage induced by UV. In this study, it was found that Se was able to protect the cells from iHg induced cell membrane damage. Similar

result was reported by Hossain et al. (2018) as Se provided protection on cell membrane integrity in PC12 cells from cadmium-cytotoxicity.

The ERK and Akt/mTOR signaling pathways are known to play crucial roles in the transmission of proliferative signals from membrane bound receptors and convey this information through interactions with various other proteins to the nucleus to control gene expression (Steelman et al. 2011). It was found that, simultaneous exposure of Se and iHg in PC12 cells upregulated mTOR, p-mTOR, akt, and ERK1 protein expressions (Fig. 4.7). These results indicated a probable involvement of mTOR/akt pathways in Se protection in toxicity. Studies have been reported to provide Se-protection in PC12 via upregulating mTOR in cadmium toxicity (Hossain et al., 2018) and arsenic toxicity (Rahman et al., 2018). Rahman et al. (2018) also reported Se induced protection in arsenic toxicity by overexpression of akt. Hossain et al. (2018) revealed that Se induced upregulation of pro-survival protein ERK1 in cadmium toxicity.

In addition, to the upregulation of pro-survival proteins, in this study, down regulation of pro-apoptotic proteins cleaved caspase 3 and cytochrome c was observed. Similarly, Ren et al. (2016) reported that Se induced inhibition of apoptosis via downregulation of caspase 3 expression. Se influenced downregulation of cytochrome c in cadmium induced toxicity for PC12 cells (Hossain et al., 2018). Finally, flow cytrometry analysis proved that Se was able to protect PC12 cells from iHg-induced apoptosis.

In summary, my study showed that Se hinders iHg-induced toxicity by its antioxidant properties reducing oxidative stress through upregulating GPx-1and GSH, influencing mTOR, akt, ERK1 and caspase 3 activation, and limiting the translocation of cytochrome c. The involvement of mTOR/akt pathway in Se-protection against iHg induced cytotoxicity in PC12 cells has never been reported before. For the first time in this study it was indicated to be involved, though further analysis is needed to reveal the details of mTOR/akt pathway in PC12 cells, as well as in other cells. Moreover, a depth of investigation in involvement of complex formation or chelation between iHg and Se is also required for clear understanding.

In conclusion, iHg has cytotoxic effects on PC12 cells and causes cell death in intrinsic apoptosis pathway. On the contrary, Se in a small concentration can protect PC12 cells from iHg-induced cytotoxicity and apoptosis via improvement of oxidative balance and antioxidants.

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Chapter 5: Protecting effects of butylated hydroxytoluene on inorganic mercury induced cytotoxicity in PC12 cells and underlying molecular mechanisms

Abstract

Mercury (Hg) is a heavy metal, well-known for its dangerous health effects on human and studied in different cell lines. Butylated hydroxytoluene (BHT) is a phenolic component generally consumed as a food additive as an antioxidant. However, the cellular mechanisms of BHT induced antioxidant properties against heavy metals-influenced toxicity are little studied. It was hypothesized that BHT has a regulatory effect on Hg induced cytotoxicity. The objective of this research was to assess the protecting effects of BHT against iHg-toxicity in PC12 cells, where cells were treated with/without $HgCl_2$ (Hg^{2+}) (5 µM) and BHT (100 µM) for 48 h and analyzed further. Cells treated by iHg caused a significant reduction of cell viability, cell membrane damage, glutathione reduction, DNA degradation. Moreover, iHg treatment caused suppressed expressions of p-akt, akt, mTOR, ERK1, nrf2, HO-1, Bcl-xL, caspase 7 and caspase 3 expressions; and elevated expressions of p53, bax, cytochrome c and active caspase 3. However, BHT and Hg^{2+} co-exposure showed prevention against iHg^{2+}-influenced toxicity by improving cell viability, LDH release, DNA damage and GSH content. Additionally, BHT co-treatment inverted the expressions of akt, p-akt, mTOR, ERK1, Nrf2, HO-1, caspase 7, caspase 3, cleaved caspase 3 and cytochrome c. These findings proved that BHT inhibits Hg^{2+}-mediated toxicity via enhancing antioxidant defense, and hindering toxicity and intrinsic apoptosis.

5.1. Introduction

Mercury (Hg) is a universal environmental pollutant which causes a broad spectrum of fatal health effects. Hg is marked as the third in most dangerous and hazardous toxic materials, by the Agency for Toxic Substances and Disease Registry (ATSDR). Severe Hg pollution in the environment (atmosphere, lake water, lake sediments and catchment soils, soil feeding termites) of anthropogenically-disturbed sites (Zhang et al., 2019; Cheng et al., 2020; Diouf et al., 2019) and dreadful contaminations in food sources (fresh and marine fish, rice, clam) (Yoshino et al., 2020; Xu et al., 2020; Sıkdokur et al., 2020) across the world have been reported. Three forms of Hg: elemental, inorganic and organic, can be exposed to human. Hg exposure typically occurs by inhalation or ingestion. Major sources of Hg are industrial emissions, fresh water and

ocean fishes, and dental amalgam. For many years, inorganic Hg has been using in various medications, germicidal soaps, teething powders, and skin care products (Guzzi and Porta, 2008). Hg crosses blood brain barrier (BBB) and can cause irreversible effect on the central nervous system. All forms of Hg can cause neurological alteration, cognitive and motor dysfunction, memory loss, behavioral disorders, immune and kidney defects, autism and neurodevelopmental difficulties (Rahman et al., 2019). Moreover, studies have been shown to induce toxicity by Hg in vivo (Goering et al., 2000; Aragão et al., 2018) and in vitro (Shenker et al., 2002; Kaur et al., 2006; Teixeira et al., 2018; Hossain et al., 2020). Hg was affirmed to induce oxidative stress and generate reactive oxygen species (ROS) in vivo and in vitro (Shenker et al., 2002; Aragão et al., 2018).

Butylated hydroxytoluene (BHT) is a widely recognized antioxidant, which was primarily used as a food additive, and also in other industries as industrial additive/preservative, personal care product, household product constituents, plastic/rubber components and medical/research uses. It is a hindered phenolic compound, also well-known as 2,6-di-tert-butyl-p-cresol or 2,6-di-tert-butyl-4-methylphenol. It is not found in nature, but produced from p-cresol and isobutylene synthetically (Branen, 1975). It is categorized by the United States Food and Drug Administration (FDA) as "Generally Recognized As Safe". Depending on the food fat content, a total of 0.02% of BHT is permitted (EFSA, 2012) and is one of the most frequently consumed antioxidants in fats-containing-foods (Yehye et al., 2015). By donation of hydrogen to free-radicals, it can prevent lipid oxidation (Babich, 1982).

Several studies have been indicated BHT induced protective effects from metals (ferric ion (Ko and Godin, 1990), cadmium (Peters et al., 1995), chromium (Pourahmad and O'Brien, 2001), copper (Hosseini et al., 2014)) and other toxic compounds (copper oxide (Assadian et al., 2017), mancozeb (Tsang and Trombetta, 2007), melatonin (Gulcin et al., 2003)) induced toxicity in vivo and in vitro, though details mechanisms were not reported yet. Studies have indicated that the antioxidant property of BHT is the major cause of BHT-protection against reactive oxygen species (ROS) induced toxicity. Very few studies are present evaluating the protective effects of BHT against metals or heavy metals induced cytotoxicity and the preventive role of BHT in the cells. The exact molecular mechanisms have not been elucidated yet. Moreover, because of the widespread usage of BHT, it is necessary to study about the safety of this antioxidant and critical evaluation of the safe limit of BHT. Most importantly, an effective solution to defend cells from

Hg induced severe toxicity is very crucial and urgent. Therefore, to evaluate the ameliorative effects of BHT against iHg induced toxicity has been studied. Here, it was hypothesized that BHT has a protective role on iHg-toxicity. The bio-molecular mechanisms involved in these protective effects were also investigated in the light of antioxidant property of BHT. In this research, PC12 cells were used as a model cell line for toxicity investigation (Hossain et al., 2018). To achieve the hypothesis, for the very first time, the protective effects of BHT were evaluated on iHg mediated toxicity in PC12 cells using various toxicity analysis methods.

5.2. Methodology

5.2.1. Chemicals

DMEM cell culture media was purchased from Sigma-Aldrich, USA. PC12 cells were bought from American Type Culture Collection (USA and Canada). BHT and $HgCl_2$ were obtained from MP Biomedicals, LLC (France) and Wako Pure Chemical Corporation (Japan), respectively. Polyclonal antibodies against p-mTOR (Cosmo Bio Co.), mTOR (2972, Cell Signalling Technology), Heme oxygenase 1 (GTX101147, GeneTex), ERK1 (610031, BD Transduction Laboratories), phosphorylated akt (9267, Cell Signalling Technology), akt (4691, Cell Signalling Technology), Nrf2 (PM069, Medical and Biological Laboratories Co., Japan), p53 (2524, Cell Signalling Technology), Bax (2772, Cell Signalling Technology), Bcl-xL (612011, BD Transduction Laboratories), Cas 7 (610812, BD Transduction Laboratories), Cas 9 (9508, Cell Signalling Technology), cleaved caspase-3 (NB100-56113SS, BioDynamics Laboratory Inc.), caspase-3 (GTX110543, GeneTEX), LC3 (83506, Cell Signaling Technology), β-actin (GeneTEX), were bought. Cytochrome c release Apoptosis Kit was bought from Calbiochem®. All chemicals used in this study were of analytical grade.

5.2.2. Cell culture

PC12 cells were cultured and maintained as described in previous studies Hossain et al., 2018; 2020). DMEM supplemented with 10% FBS were used to culture the cells and cells were maintained at 37°C for 48 h. The medium was substituted with serum/serum-free DMEM with/ without different concentrations of $HgCl_2$ (Hg^{2+}) (0-5 μM) and butylated hydroxytoluene (BHT) (0-1000 μM), separately. The IC50 value for $HgCl_2$ was analyzed. Upon analyzing IC50 value (4.37 μM) of Hg, the final concentration for Hg^{2+} was decided as 5 μM for the co-exposure. 100 μM of BHT was selected as the final concentration for the co-treatment, because till 100 μM

BHT did not show any toxic effect on the cells. The final combinations were decided as 5 μM of Hg^{2+} and 100 μM of BHT for the co-treatment. Therefore, cells were treated with/without BHT (100 μM), iHg (5 μM), or BHT (100 μM) and iHg (5 μM) together for 48h and further tested for various cytotoxicity assessment analysis.

5.2.3. Cell viability analysis

The viability of the cells was dignified by trypan blue exclusion analysis. Cells were incubated with/without BHT (100 μM) and iHg (5 μM), or co-incubated with BHT (100 μM) and iHg (5 μM) for 48h in the DMEM supplemented with FBS (10%). After the incubation, cells were harvested and washed using 1x PBS buffer. Cells were tainted with trypan blue dye. To measure the stained and non-stained cells, an automated cell counter (Bio-Rad, USA) was employed. These experiments were executed at least ten times to justify reliability.

5.2.4. Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase activity analysis kit (Promega, USA) was used to assess the membrane integrity of the cells after 48h of treatment with/without BHT (100 μM), iHg (5 μM) or BHT (100 μM) and iHg (5 μM) together. After the 48h of incubation of the cells with the various treatments, the culture medium of the cells was measured for LDH activity, as stated in previous research (Hossain et al. 2018). Reproducibility was verified by performing these analyses four times.

5.2.5. Genomic DNA extraction

Cells were incubated with/without BHT (100 μM), iHg (5 μM) or BHT (100 μM) and iHg (5 μM) together for 48h, and then were harvested and washed with 1x PBS buffer. The technique described by Hossain et al. (2018) was used to extract DNA from the cells with High Pure PCR Template preparation kit (Germany)

5.2.6. Electrophoresis of genomic DNA

The DNA was extracted (as described by Hossain et al. 2018) and the intact genomic DNA of the cells was assayed by agarose gel electrophoresis assay. Isolated DNA of the cells treated or co-treated with/without BHT (100 μM) and iHg (5 μM) were measured using 1.5% agarose gel electrophoresis as defined by Hossain et al. (2018). The fluorescence intensity of intact DNA

was quantified using 'Quantity one' software. The reproducibility was ensured by performing this experiment at least four times.

5.2.7. Free sulfhydryl (SH) levels analysis

Intracellular free sulfhydryl (SH) or glutathione levels assay was measured as explained in Hossain et al. (2018). PC12 cells were exposed or co-exposed with/without BHT (100 μM) and iHg (5 μM) for 48h, and then were harvested and washed with 1x PBS buffer. The protein extraction and free sulfhydryl (SH) level measurement were performed as stated by Hossain et al. (2018). The assay was conducted three times to verify reliability.

5.2.8. Flow cytometry study

Flow cytometry was used to assess the cell death process using Annxin V-FITC apoptosis detection kit (BioVision, USA). Cells were incubated or co- incubated with/without BHT (100 μM) and iHg (5 μM) for 48h, and then flow cytometry was done as explained by Hossain et al. (2020). The experiments were performed three times for reliability.

5.2.9. Western blot for protein expression determination

Cells were treateded with/without BHT (100 μM) and iHg (5 μM), or treated with BHT (100 μM) and iHg (5 μM) together for 48h. After incubation, cells were harvested and washed by 1x PBS. As described in Hossain et al. (2018) protein extraction from the cells and electrophoresis were done. The proteins were relocated into a nitrocellulose membrane with the use of a semidry blotting system. The proteins were blocked in the membranes using skimmed milk, washed with washing buffer and treated with the primary antibodies for 1h. The membranes were washed, treated with the secondary antibody on a shaker for 1h at room temperature and washed for 5 times with washing buffer. Enhanced chemiluminescence was used to detect the protein bands and; those bands were evaluated by a ChemiDoc XRS (Bio-Rad, USA) and were quantified using 'Quantity one' software. The reproducibility was ensured by performing these experiments at least four times.

5.2.10. Statistics

Data were presented as the mean ± standard error of mean (SEM) of independent experiments (at least three). Analysis was performed using ANOVA, followed by unpaired Student's t-test for statistical analysis. Either $p<0.05$ or $p<0.01$ difference was reflected as significant statistically.

5.3. Results

5.3.1. Assessment of cell viability

To examine the BHT-effect on cells, cells were incubated with various concentrations (0-1000 µM). Fig. 5.1A shows the cell viability when incubated with 0-1000 µM of BHT. Up to 100 µM of BHT concentrations the viability did not show any significant variance from the control cells (treated with no treatment). But over 100 µM of BHT concentrations, the viability of the cells were drastically ($p<0.01$) reduced in opposition to the control group. This result indicated that upto 100 µM concentration of BHT is not toxic or harmful to PC12 cells. For this reason 100 µM of BHT was chosen for the combined treatment with iHg.

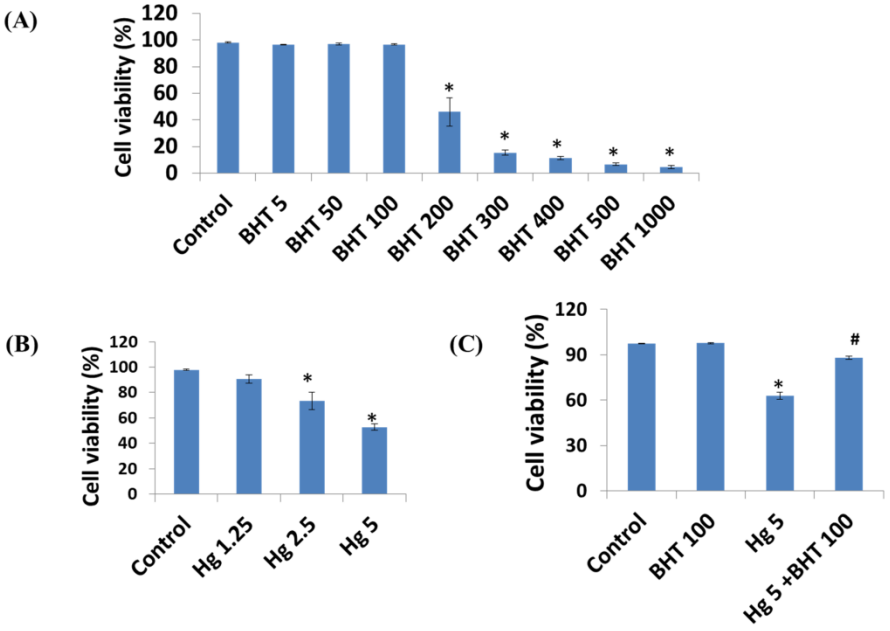

Figure 5.1: (A) Cell viability of PC12 cells cultured in medium with 0-1000 µM of BHT, (B) Cell viability of PC12 cells cultured in medium with 0-5 µM of iHg, (C) Cell viability of PC12 cells co-exposed with BHT (100 µM) and iHg (5 µM) for 48 h incubation. Error bars indicate mean ± SEM (n= at least 6). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group.

For selecting the iHg concentration for the combined treatment, PC12 cells were incubated with various concentrations of iHg. Fig. 5.1B displays the viability of cells incubated with 0, 1.25, 2.5 and 5 µM of iHg concentrations. Cell groups treated with 1.25 µM of iHg did not show any

significant ($p<0.01$) difference in comparison with the control group, whereas cell groups treated with 2.5 and 5 μM of iHg, both displayed significant ($p<0.01$) cell viability loss in opposition to the control group, with IC50 value of 4.37 (approximately 5). For this reason, 5 μM of iHg was chosen for the combined treatment with BHT.

To evaluate the ameliorative effect of BHT on iHg induced toxicity in cells, cells were incubated with/without BHT (100 μM) and iHg (5 μM), or co-treated with BHT (100 μM) and iHg (5 μM) for 48h; and cell viability assay was performed. Fig. 5.1C displayed the viability of cells treated or co-treated with/without BHT (100 μM) and iHg (5 μM). The viability of the cells incubated with BHT (100 μM) alone did not expressed any significant difference from the control group, but the cells incubated with only iHg (5 μM) showed a significant ($p<0.01$) cell viability drop in opposition to the control. Interestingly, the cells treated with BHT (100 μM) and iHg (5 μM) together displayed a significant ($p<0.01$) rise in cell viability when compare with the cells treated with only iHg (5 μM). These findings suggested that BHT has a protective regulatory effect on iHg-toxicity in PC12 cells.

5.3.2. Cell-membrane integrity

To assess the cytotoxicity of iHg, cell-membrane damage was detected by trypan blue assay. Fig 5.2A showed the pictures of cells treated or co-treated with/without BHT (100 μM) and iHg (5 μM) for 48h and then stained with trypan blue. Here, red colored cells are indicating the cell membrane damaged cells and green colored cells are indicating cell membrane intact cells. The cells incubated with only iHg, presented a significant increase of red colored (cell- membrane damaged or dead) cells, in opposition to control cells. However, the cells co-incubated with BHT (100 μM) and iHg (5 μM), showed a significant decrease of red colored cells, when compared with the cells treated with only iHg (5 μM).

To double check the modulation of cell-membrane damage, another cytotoxicity assessment method LDH activity assay was conducted. Fig 5.2B showed the LDH activity in the culture media incubated or co-incubated with/without BHT (100 μM) and iHg (5 μM) for 48h. The activity of LDH in the cells incubated with only iHg (5 μM), resulted in a significant rise ($p<0.01$) in opposition to that of the control group, which indicating a severe cell membrane damage. But the activity of LDH when co-treated with BHT (100 μM) and iHg (5 μM), showed a significant ($p<0.05$) drop in opposition to the cells incubated with iHg (5 μM) alone; which

indicating improvement in the cell-membrane. These findings together with cell viability assay evidenced that BHT protects cells from iHg induced cytotoxicity and membrane damage.

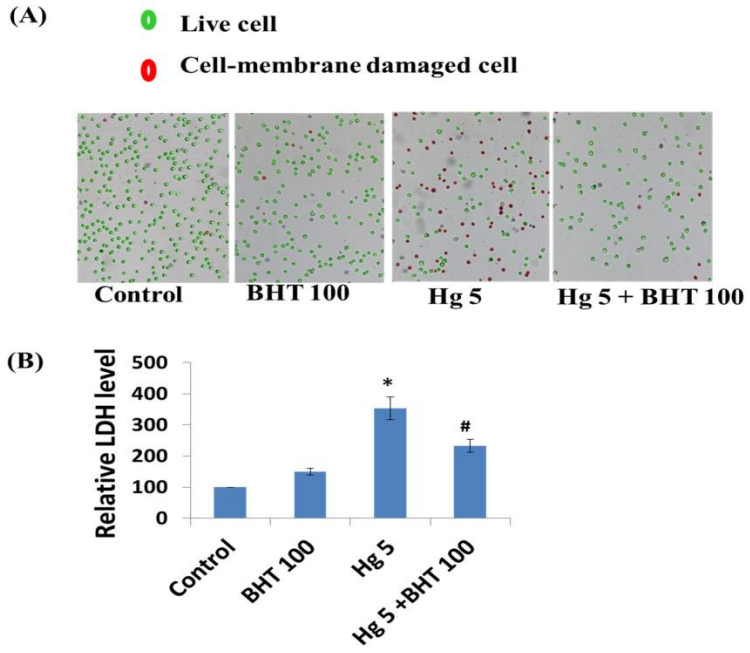

Figure 5.2: (A) Detection of cell wall damage in the cultured cells using trypan blue staining when cells were treated with/without BHT (100 µM), iHg (5 µM), or BHT (100 µM) and iHg (5 µM) together for 48 h. (B) LDH levels in the cultured medium for PC12 cells treated with/without BHT (100 µM), iHg (5 µM), or BHT (100 µM) and iHg (5 µM) together for 48 h. Each column is shown as mean ± SEM, n=5. * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group.

5.3.3. DNA degradation

Genomic DNA extraction and agarose gel electrophoresis experiment was implemented to evaluate the protective effect of BHT on iHg influenced cytotoxicity. Fig 5.3A showed the image of electrophoresed extracted DNA of the cells incubated or co- incubated with/without BHT (100 µM) and iHg (5 µM); and Fig. 5.3B showed the quantification of the DNA intensity. The amount of relative intact DNA was significantly ($p<0.05$) reduced in the cells incubated with only iHg (5 µM) comparing to those control cells. On the other hand, the cells co-treated with BHT (100 µM) and iHg (5 µM) together, revealed a significant ($p<0.05$) increase in the relative intact DNA level in opposition to the cells incubated with only iHg. These findings suggested that BHT co-

treatment with iHg, improves the intact DNA and blocked iHg encouraged DNA-degradation in cells.

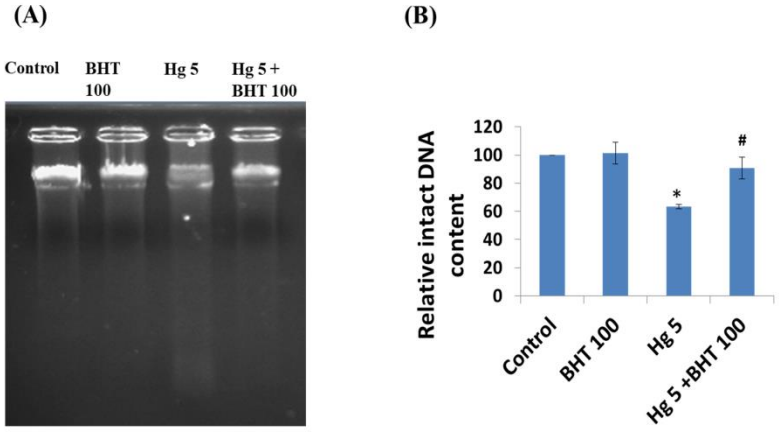

Figure 5.3: (A) Agarose gel electrophoresis of DNA isolated from PC12 cells treated with/without BHT (100 µM), iHg (5 µM), or BHT (100 µM) and iHg (5 µM) together for 48 h. Intensity of band obtained from DNA in the cells treated was shown below the electrophoresis band (B). Error bars are mean ± SEM (n=4). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group.

5.3.4. GSH levels

Free SH group or glutathione level in the cells is a vital element for antioxidant defense mechanisms. Reduced glutathione (GSH) is altered to its oxidized form (GSSG) for defending the cells from ROS influenced oxidative stress. To assess the antioxidant defense state of the cells and the contribution of ROS mediated stress in iHg-treated cells, intracellular GSH level was evaluated. Fig. 5.4 showed the GSH level in cells incubated or co- incubated with/without BHT (100 µM) and iHg (5 µM). The GSH level in the cells incubated with only iHg (5 µM) was significantly ($p<0.01$) dropped in opposition to the control cells, indicating significant upsurge in ROS amount in the cells. Again, while compared with the cells treated with only iHg (5 µM), the GSH level of the cells co-treated with BHT (100 µM) and iHg (5 µM) revealed a significant ($p<0.01$) uplift, indicating significant decrease in ROS amount in the cells. These findings suggested that iHg persuades ROS arbitrated oxidative stress and BHT protects the cells from ROS mediated oxidative stress and cell death by improving the GSH content of the cells.

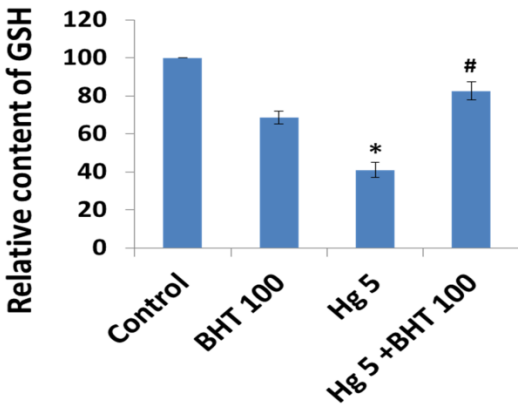

Figure 5.4: GSH contents in the cultured cells treated with/without BHT (100 µM), iHg (5 µM), or BHT (100 µM) and iHg (5 µM) together for 48 h. Error bars are mean ± SEM (n=3). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group.

5.3.5. Flow cytometry analysis for apoptosis detection

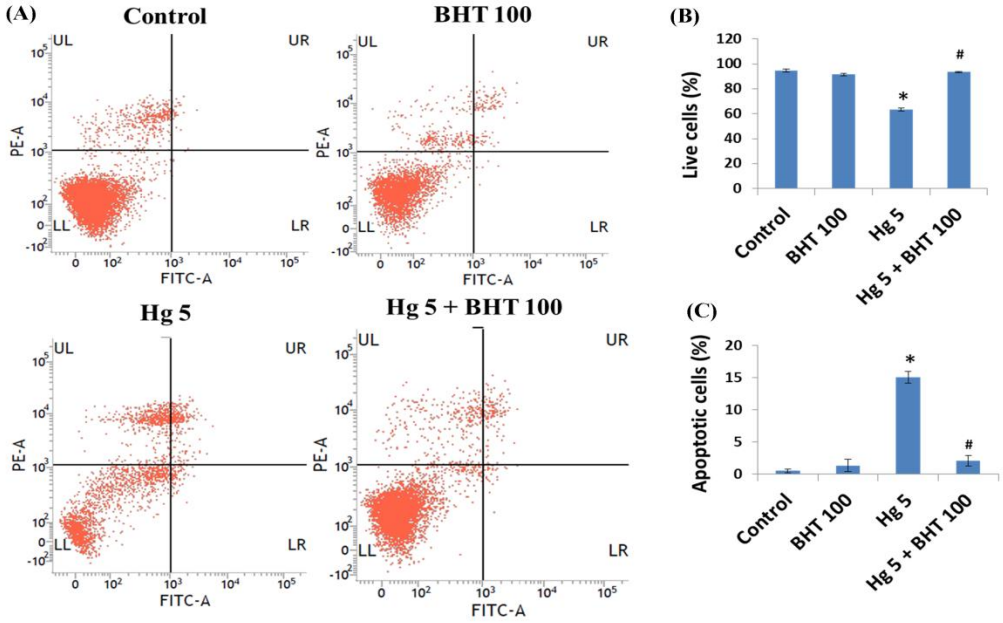

Figure 5.5: (A) Flow cytometry analysis of cultured PC12 cells, strained with Annexin V-FITC/PI after treatment with/without BHT (100 µM), iHg (5 µM), or BHT (100 µM) and iHg (5 µM) together for 48 h; and percentage of live cells (B) and apoptotic cells (C). Error bars are mean ± SEM (n=3). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group.

To know about the cell death mechanisms flow cytometry analysis was employed. The cells exposed to only iHg (5 μM), presented a significant growth ($p<0.05$) in apoptotic cells in opposition to the cells treated with no treatment (Fig 5.5). Again, the cells co-treated with BHT (100 μM) and iHg (5 μM), disclosed a significant decrease in apoptotic cells in opposition to the cells treated with only iHg (5 μM). These findings suggested that BHT provides protection from iHg mediated toxicity and inhibited apoptotic cell death.

5.3.6. Protein expressions of the cells

The molecular mechanisms involved in BHT mediated protection and iHg induced toxicity in cells were investigated using western blotting analysis. The protein samples were extracted from the cells treated or co-treated with/without BHT (100 μM) and iHg (5 μM) and western blotting was performed. The protein expressions of β-Actin, p-akt, akt, p-mTOR, mTOR, ERK1, Nrf2, HO-1, p53, Bax, Bcl-xL, caspase-7, cleaved caspase-3, caspase-3, cytochrome c, and LC3 were shown in fig 5.6 and fig 5.7.

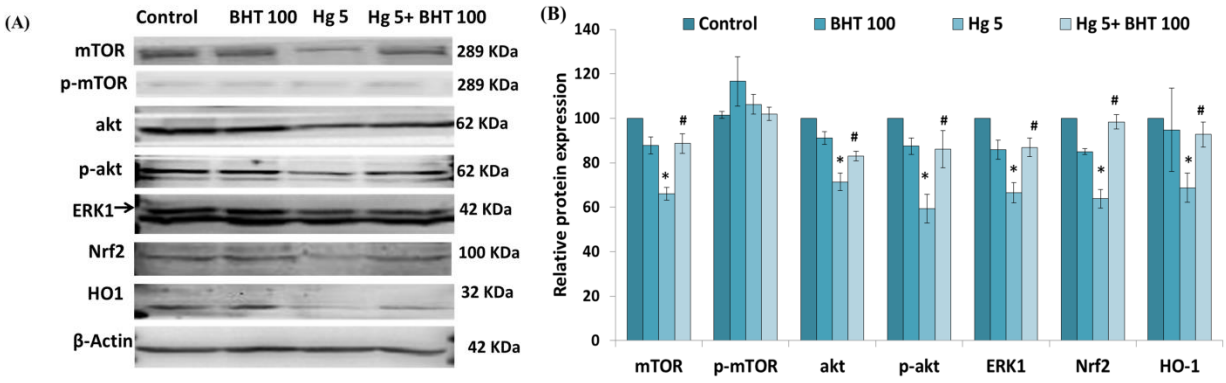

Figure 5.6: (A) Relative protein expression and (B) quantification of mTOR, p-mTOR, akt, p-akt, ERK1, Nrf2, HO-1 and β-actin in the cultured cells treated with/without BHT (100 μM), iHg (5 μM), or BHT (100 μM) and iHg (5 μM) together for 48 h. Error bars are mean ± SEM (n= atleast 4). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group.

The mTOR (mammalian target of rapamycin) plays a principal regulatory role in cellular metabolism, growth, proliferation, differentiation and survival. mTOR and phosphorylated mTOR, both were investigated in this study (Fig. 5.6). The level of mTOR was significantly ($p<0.01$) downregulated in cells treated with only iHg (5 μM) in opposition to that of the control

group. Again, mTOR level was upregulated significantly ($p<0.05$) when co-incubated with BHT (100 μM) and iHg (5 μM) in opposition to the cells incubated with only iHg (5 μM). Though, the p-mTOR level did not display any significant difference between the groups.

Akt regulates cellular metabolism, survival, cell cycle, and apoptosis by regulating many downstream proteins, for example, NFkB, Bcl-2 family proteins. To investigate the involvement of akt in this study, akt and phosphorylated akt were assessed (Fig. 5.6). Both akt and p-akt protein levels in the cells incubated with only iHg (5 μM), were significantly ($p<0.01$) reduced in opposition to the control cells proteins. Inversely, both proteins in the cells co-treated with BHT (100 μM) and iHg (5 μM) were significantly increased compared to the only-iHg (5 μM) treated cells.

ERK1/2 plays a crucial role in regulating cell proliferation, differentiation, cycle progression, metabolism, transcription and survival (Roskoski, 2012). The ERK1 level in the cells incubated with only iHg (5 μM), were significantly ($p<0.01$) reduced in opposition to the control cells proteins. On the contrary, ERK1 level in the cells co-exposed to BHT (100 μM) and iHg (5 μM) were significantly increased compared to the only-iHg (5 μM) treated cells.

Nrf2 modulates the expression of antioxidant proteins e.g., Glutathione reductage (GR) and heme oxygenase-1 (HO1) which defend against oxidative damage induced by inflammation and injury. To inquiry about the contribution of nrf2-HO1 pathway, nrf2 and HO1 protein levels were checked (Fig. 5.6). The level of nrf2 was significantly ($p<0.01$) downregulated in cells exposed to only iHg (5 μM) significantly ($p<0.01$) reduced in opposition to the control cells; while the nrf2 level was upregulated significantly ($p<0.01$) in cells co-exposed to BHT (100 μM) and iHg (5 μM) in opposition to the cells treated with only iHg (5 μM). Again, while compared with the control group the HO1 level was significantly ($p<0.01$) reduced in the cells exposed to only iHg (5 μM). And comparing with the only-iHg (5 μM) treated group, HO1 level was significantly ($p<0.05$) increased in the group co-treated with BHT (100 μM) and iHg (5 μM).

The p53 protein acts a fundamental part in responding to genomic abnormalities and DNA degradation; thus could cause seizure of cell cycle and apoptosis. The p53 protein level was abruptly ($p<0.01$) increased in cells incubated with only-iHg (5 μM) in opposition to the control cells, whereas p53 level was significantly ($p<0.05$) decreased in cells co-incubated with BHT (100 μM) and iHg (5 μM) together as compared with cells incubated with only-iHg (5 μM).

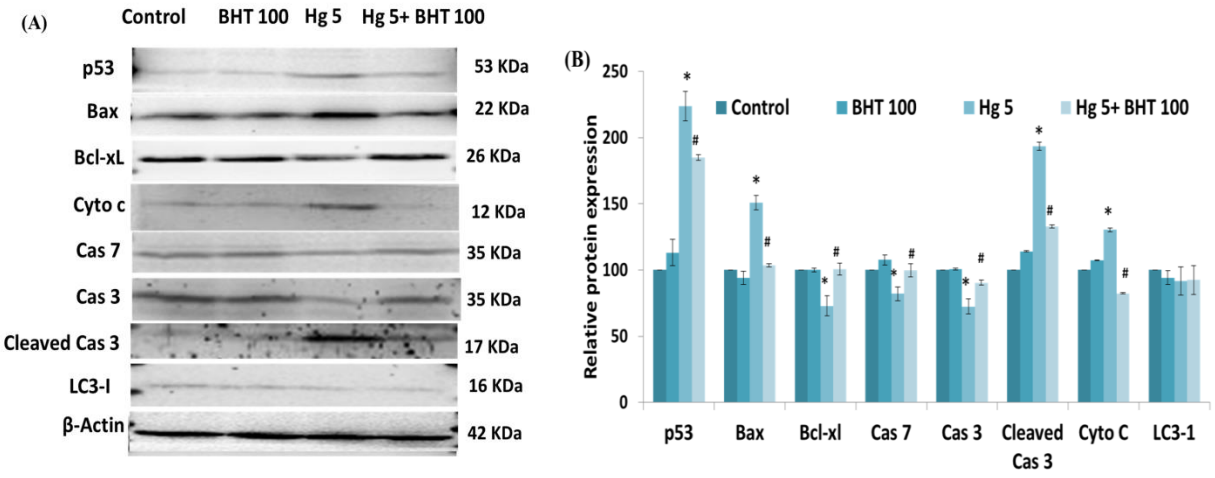

Figure 5.7: (A) Relative protein expression and (B) quantification of p53, Bax, Bcl-xL, cytochrome c, Caspase 7, caspase 3, cleaved caspase 3, LC3-I and β-actin in the cultured cells treated with/without BHT (100 µM), iHg (5 µM), or BHT (100 µM) and iHg (5 µM) together for 48 h. Error bars are mean ± SEM (n= atleast 4). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group.

The Bcl-2 family is well known regulatory proteins of programmed cell death apoptosis, which consists of pro-apoptotic or anti-apoptotic members that either promote or inhibit apoptosis and control apoptosis. These proteins regulate the permeability of mitochondrial outer layer and cytochrome c discharge into cytosol from mitochondria, which is a basic part of intrinsic apoptosis of the cells. In this research, with the cytochrome c level in the cytosol, pro-apoptotic Bcl-2 family protein Bax and anti-apoptotic Bcl-2 family protein Bcl-xL have been studied (Fig 5.7). The anti-apoptotic Bcl-xL level was significantly ($p<0.05$) downregulated in only-iHg (5 µM)-exposed cells in opposition to the control cells. Contrarily, Bcl-xL level was significantly ($p<0.05$) upregulated when cell treated with BHT (100 µM) and iHg (5 µM) together as compared with the only-iHg (5 µM)-treated cells.

Moreover, the pro-apoptotic Bax level was significantly ($p<0.01$) upregulated in only-iHg (5 µM)-treated cells while compared with that of the control cells. Inversely, Bax level was significantly ($p<0.01$) down regulated when cell treated with BHT (100 µM) and iHg (5 µM) together as compared with the only-iHg (5 µM)-treated cells. Similarly, cytochrome c level in the

cytosol was significantly ($p<0.01$) upregulated in cells treated with only-iHg (5 μM) while compared with that of the control cells; and indicating intrinsic apoptosis occurred in only-iHg (5 μM) treated cells. Then again, cytochrome c level was significantly ($p<0.01$) downregulated when cell treated with BHT (100 μM) and iHg (5 μM) together as compared with the only-iHg (5 μM)-treated cells; indicating inhibition of intrinsic apoptosis by BHT co-treatment.

Caspase 7 is an effector caspase family protein that executes apoptosis. Activation of cas 7 is a sequential step of apoptosis induction. Cas 7 protein level was significantly ($p<0.01$) downregulated in cells exposed to only iHg (5 μM) in opposition to that of the control group; indicating cas 7 activation in only-iHg (5 μM)-treated cells. Again, cas 7 level was upregulated significantly ($p<0.05$) when co-exposed to BHT (100 μM) and iHg (5 μM) in opposition to the cells treated with only iHg (5 μM); indicating inhibition of Cas 7 activation in co-treated group cells. Similarly, another vital caspase family protein Cas 3 and its activated form were also investigated in this study (Fig. 5.7). Cas 3 protein level was significantly ($p<0.01$) downregulated in only-iHg (5 μM)-exposed cells in opposition to the control cells; indicating cas 3 activation in only-iHg (5 μM)-treated cells. Again, cas 3 level was significantly ($p<0.05$) upregulated when cell treated with BHT (100 μM) and iHg (5 μM) together as compared with the only-iHg (5 μM)-treated cells. The cleaved or activated cas 3 level was significantly ($p<0.01$) increased in cell group treated with iHg (5 μM)-only in opposition to the control group and confirmed apoptosis. Inversely, activated cas 3 level was significantly ($p<0.05$) decreased in cell group co-exposed with BHT (100 μM) and iHg (5 μM) compared to the group treated with only- iHg (5 μM) and confirmed blockage of apoptosis.

LC3 is a protein related to another programmed cell death autophagy, in which LC3-I is converted to LC3-II to conjugate autophagosomes. To investigate autophagy LC3 protein was assessed (Fig. 5.7), though only LC3-I was observed while LC3-II was absent indicating no involvement of autophagy. Fig. 5.8 depicts a graphic diagram of BHT-induced protection on iHg mediated toxicity in PC12 cells.

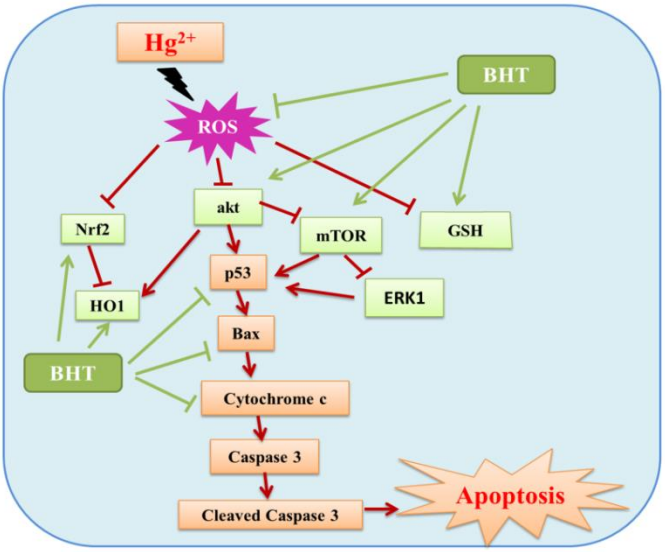

Figure 5.8: Schematic diagram of BHT-protection on iHg-induced cytotoxicity in PC12 cells

5.4. Discussion

There are approximately six thousand scientific articles discussing exclusively on BHT and its derivatives-induced antioxidant activities. BHA is a mixture of 15% 2-tert-butyl-4-hydroxyanisole (II) and 85% 3-tertbutyl-4-hydroxyanisole (III) (Francois et al., 2012). Recently, BHT is comprehensively applied in the food industry, especially in low-fat foods, packaging materials and fish products (Yehye et al., 2015). After accumulation from food, BHT is primarily metabolized in liver and a small amount metabolized in lungs; and finally excreted via urine (Babich, 1982). The antioxidant property of BHT has been studied by various researchers (Lambert et al., 1996; Dawidowicz and Olszowy, 2011) indicating BHT as an inhibitor of free radical-chain reactions (Yehye et al., 2015). Numerous studies have been published on other antioxidants, where BHT has been used as an antioxidant standard (Dawidowicz and Olszowy, 2010; Sánchez-Vioque et al., 2012). In this study the antioxidant properties of BHT have been studied against a heavy metal induced toxicity (e.g., inorganic Hg mediated cytotoxicity) in PC12 cells using several cytotoxicity assessment assays.

Hg is an extremely toxic substance to human health, which has been evidenced to be hazardous even at a low concentration. In this research, relatively higher concentration was used to induce toxicity in PC12 cells. Cells exposed to iHg concentration (5 μM) showed a significant cell viability reduction (Fig. 5.1), compromised cell membrane integrity (Fig. 5.2), genomic DNA

degradation (Fig. 5.3), reduced glutathione amount diminution (Fig. 5.4). Similar results were also found by previous researches. Kim et al. (2003) reported similar iHg induced cell viability loss, cell membrane damage (LDH release) and blocked DNA proliferation in murine T and B lymphoma cells. Lee et al. (2001) described iHg influenced cell viability loss, ROS generation and GSH content drop in glioma cells. In the previous research (Hossain et al., 2020), iHg has been proved to be apoptogenic to PC12 cells. Similarly, present research has appeared to encourage apoptosis in PC12 cells via mitochondrial pathway (Fig 5.5, 5.6 and 5.7). iHg caused downregulating p-akt, akt, mTOR, ERK1, nrf2, HO1, Bcl-xL, inactivate caspase 7 and inactivated caspase 3 expressions; whereas resulted in upregulating protein expressions of p53, bax, cleaved caspase 3 and cytochrome c (Fig 5.6 and 5.7) synergistically, demonstrating oxidative stress and ROS generation. Kim et al. (2003) reported similar iHg induced apoptosis in lymphoma cells. Teixeira, et al. (2018) showed iHg mediated oxidative stress and apoptosis in motor cortex. It was demonstrated here that iHg (5 μM) induces cytotoxicity and cell death in PC12 cells via compromising cellular antioxidant defense associated with oxidative stress.

To protect cells from heavy metal induced toxicity, various antioxidants (selenium, dihydrolipoic acid, zinc) have been used for limiting ROS generation and oxidative stress (Hossain et al., 2018; 2020; Rahman et al., 2017). A number of researches have been shown the possibility of BHT-protection upon heavy metals and other toxicants-induced toxicity (Hosseini et al., 2014; Al-Malki, 2010; Assadian et al., 2017; Tsang and Trombetta, 2007; Gulcin et al., 2003). In this research, BHT (100 μM) co-treatment with iHg (5 μM) showed significant improvement in cell viability, genomic DNA and cell membrane integrity (LDH level decrease) Similarly, Tsang and Trombetta (2007) reported BHT-improved cell viability from mancozeb-induced cytotoxicity in rat hippocampal astrocytes. Lison et al. (1995) demonstrated in a macrophage culture model, BHT decreased the LDH release caused by the cytotoxicity of hard metal particles (cobalt metal and carbide particles). Again, Pourahmad and O'Brien (2001) showed that BHT prevented dichromate induced lipid peroxidation in isolated rat hepatocytes. Assadian et al. (2017) showed BHT mediated protection from lipid peroxidation caused by copper oxide nanoparticles influenced toxicity on human blood lymphocytes. Ko and Godin (1990) demonstrated that BHT inhibited the ferric ion-ininfluenced peroxidation of erythrocyte membrane lipids. In this study, reduced glutathione content was improved by BHT (100 μM) co-treatment with iHg (5 μM) indicating ROS formation. Similar to this results, Assadian et al. (2017) showed BHT induced

glutathione content increase in human blood lymphocytes after copper oxide nanoparticles-treatment. Pourahmad and O'Brien (2001) showed that BHT prevented dichromate induced reactive oxygen species (ROS) formation in isolated rat hepatocytes. It was assumed that the mechanism of BHT associated antioxidant properties consist of the quenching of ROS and lipid soluble radicals (Yehye et al. 2015).

Antioxidant activity is extensively utilized as factor to illustrate substances with the property of neutralizing free radicals, by which the substance is able to protect a biological system from harmful oxidation. Scavenging property of BHT has been scrutinized critically (Yehye et al. 2015; Gulcin et al., 2003) and considered the major cause of antioxidant activity.

In this study, the proteins responsible for cellular metabolism, cell survival and cell cycle, akt, p-akt, mTOR, ERK1 were rejuvenated by BHT exposure against iHg induced toxicity. One of the major cell survival pathway, nrf2-HO1 is also potential to be involved in BHT induced protection, as p-akt, nrf2 and HO1 were upregulated upon BHT co-exposure with iHg. Similar nrf2 and HO1 protein upregulation was also found in iHg-induced toxicity with another antioxidant dihydrolipoic acid pretreatment (Hossain et al., 2020). In this study, BHT was able to inhibit the pro-apoptotic proteins p53, bax, caspase 7, cytochrome c and caspase 3, and regulated the anti-apoptotic proteins mtor, akt, ERK1, Bcl-xL, preserving mitochondrial membrane potentiality and modulated apoptosis caused by iHg in PC12 cells. Hosseini et al. (2014) reported BHT mediated protection from lipid peroxidation caused by copper toxicity on isolated liver mitochondria, describing mitochondrial membrane potential loss. Again, Assadian et al. (2017) showed BHT improved mitochondrial membrane potential in human blood lymphocytes after copper oxide nanoparticles-treatment.

In summary, consistent with other reports, this study elucidated that iHg induced cell viability drop, cell membrane damage, genomic DNA degradation, glutathione amount decrease, with downregulation of cytoprotective proteins mTOR, akt, ERK1, nrf2, HO1, Bcl-xL, and upregulation of apoptosis inducing proteins p53, bax, caspase 3 and cytochrome c. Inversely, BHT co-treatment with iHg protected the cells from iHg-cytotoxicity by modifying cell viability loss, inhibiting cell membrane damage, protecting genomic DNA, limiting glutathione decrease, with downregulation of apoptosis inducing proteins p53, bax, caspase 3 and cytochrome c, and subsequently upregulating survival regulating proteins mTOR, akt, ERK1, nrf2, HO1, Bcl-xL. It appeared to be a good antioxidant against Hg induced toxicity and cellular damages.

5.5. Conclusion

Our results demonstrated that inorganic mercury is a highly toxic to PC12 cells, whereas butylated hydroxytoluene can protect PC12 cells from inorganic Hg induced toxicity. The protection provided by BHT in iHg mediated toxicity is via its antioxidant properties. Further analysis is needed to investigate the chelation capability of BHT antioxidant. The results obtained from this research can be used in pharmaceuticals and pharmacological use of BHT as a therapeutic agent against environmental toxic substances. This is the first study directly enlightening the ameliorative effect of BHT against iHg-toxicity on cells.

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Chapter 6: Zinc-pretreatment triggers nrf2-mediated protection against inorganic mercury-induced toxicity and intrinsic apoptosis in PC12 cells

Abstract

Mercury (Hg) is a widely used metal in commercial purposes, and poses environmental problems with severe human health effects; whereas zinc (Zn) is an essential micronutrient with a wide range of antioxidant properties. The purpose of this research was to investigate the effect of Zn on inorganic Hg-induced cytotoxicity in the PC12 cells. The cells were exposed to $HgCl_2$ (5 µM) for 48h with/without 1 h prior $ZnCl_2$-treatment (100 µM) and deliberated for further analysis. After 48 h of incubation with only Hg^{2+}, the cell showed reduced cell viability, compromised cell membrane, increased DNA degradation, depleted glutathione level, and drastically increased apoptotic cells. Subsequently, Hg^{2+}-treated cells demonstrated a significant downregulation of akt, mTOR, ERK1, nrf2, HO1, bcl2, bcl-xL, and upregulation of p53, bax, cytochrome c and cleaved caspase 3, indicating intrinsic apoptosis induction. However, cells pretreated with Zn^{2+} before Hg^{2+}-exposure showed a significant improvement in cell viability, cell membrane, DNA damage, glutathione level and apoptotic cells, with a significant upregulation in mTOR, akt, p-akt, ERK1, nrf2, HO1, bcl2 and bcl-xL, and downregulation in p53, bax, cytochrome c and cleaved caspase 3, indicating inhibition of intrinsic apoptosis by Zn^{2+}-pretreatment. The findings suggested that Zn^{2+}-pretreatment not only improves glutathione content but also induces activation of nrf2-HO1 pathway, which would tend to suppress mercury cytotoxicity and inhibit intrinsic apoptosis induced by Hg.

6.1. Introduction

Mercury (Hg) is an environmental pollutant, classified as one of the most hazardous substances on earth because of its known toxicity to human. It exists in several forms: Hg vapor (Hg^0); inorganic Hg, mercurous (Hg^+) or mercuric (Hg^{2+}) salts; and organic mercury (mainly, methylmercury and ethylmercury). The biological behavior, toxicokinetics, and clinical significance of Hg vary with its form and chemical structure. Major sources of Hg are sea foods consumption, dental fillings/amalgums, vaccines containing thimerosal, occupational exposure from industries, manufacture of thermometer, fluorescence light bulb and so on (Rahman et al., 2019). Hg crosses blood brain barrier and causes numerous detrimental effects in adults (Shigematsu et al., 2000) and developing organisms (Peixoto et al., 2007) which predominantly affect the central nervous system and renal systems (Orr et al.,

2019). Detrimental health issues, for instance, neurological and behavioral disorder, abnormal motor function, compromised immunity, Alzheimer, fertility difficulties, difficulties in fetus development, abnormal offspring, autism, sensory disabilities, fatigue, renal and cardiovascular damages have been reported to cause by Hg exposure (EPA 2017). Previous in vivo and in vitro reports have demonstrated that Hg causes reactive oxygen species (ROS) formation and lipid peroxidation, indicating that the cellular damages induced by ROS (superoxide, hydrogen peroxide, hydroxyl radical, and peroxynitrite), may contribute to mercury intoxication and diseases (Lee et al., 2001; Yang et al. 2016a, 2016b; Hossain et al., 2020).

One of the most significant essential trace metals in human body is zinc (Zn), and deficiency of Zn is a worldwide nutritional problem. It is crucial for various human functions, including growth and development, neuropsychiatric and immune functions, bone metabolism, and wound healing (Marreiro et al., 2017). Zn functions as a catalyst for enzymes in DNA replication, gene transcription, and synthesis of RNA and proteins. Together with dydrolases, transferases, oxidoreductases, ligases and lyases; over 2700 enzymes contain Zn (andreini and bertini, 2012). In addition, Zn shows antioxidant properties via different pathways including metallothionein formation.

Antioxidant properties of Zn and its therapeutic role against oxidative stress and metal toxicity have been reported in vivo and in vitro (Rahman et al., 2017; Pérez and Cederbaum, 2003; Kumar et al., 2010; Franco-Vidal et al., 2008; Milton et al., 2004). Rahman et al. (2017) reported that Zn in co-exposure with cadmium protected the PC12 cells from cadmium mediated toxicity. Pérez and Cederbaum (2003) reported that Zn protects HEPG2 cells against CYP2E1-dependent toxicity via metallothionein 2A induction. Kumar et al. (2010) reported that Zn protected rat liver from arsenic-induced oxidative stress and histological changes. Ameliorative effects of Zn on pneumolysin-toxicity in rat cochlear hair cells were reported by Franco-Vidal et al. (2008). Milton et al. (2004) also demonstrated Zn protection on arsenic-influenced apoptosis in neuronal cells. Moreover, Zn-protection against Hg-induced toxicity has been also reported (Franciscato et al., 2011; Mesquita et al., 2016), although no studies were observed in cellular level. Therefore, investigation on the interaction of Zn and Hg in cellular level is necessary and exploration on the biomolecular alterations responsible for Hg-toxicity and its reversals by Zn-pretreatment is essential. Thus, for the very first time in this study the protective effect of Zn-pretreatment in inorganic mercury induced toxicity was investigated in PC12 cells. Here, PC12 cells have been used as a model cell-line for toxicity evaluation. Here it was hypothesized that Zn-pretreatment has a

regulatory role on inorganic Hg-toxicity in cells. To accomplish the hypothesis, the protective effects of Zn-pretreatment were examined against Hg mediated cytotoxicity in PC12 cells using various toxicity exploration approaches.

6.2. Methodology

6.2.1. Cell culture

PC12 cells were cultured and incubated as explained in earlier researches (Hossain et al., 2018; 2020). At 37°C, DMEM medium accompanied with 10% FBS were used to culture the cells. After 2 days, medium was replaced by medium with /without $HgCl_2$ (Hg^{2+}) concentrations (0-5 μM) and $ZnCl_2$ (Zn^{2+}) concentrations (0-500 μM), independently.

After examining IC_{50} value (4.37 μM) of Hg, the final concentration for Hg^{2+} was decided as 5 μM for the combined treatment with Zn. The final concentration of Zn for combined treatment with Hg was selected as 100 μM (because till 100 μM concentration Zn was observed to be indifferent compared to the control group cells). Upon dose selection, cells were co-treated and pretreated with Zn^{2+} (100 μM), together with Hg^{2+} (5 μM)-treatment for 48 h and later tested for a number of cytotoxicity assessments.

6.2.2. Cell viability analysis

Cells were incubated with/without Zn^{2+} (100 μM)-pretreatment (1 h) followed by Hg^{2+} (5 μM)-exposure. After 48h of incubation, the cells mortality was examined using trypan blue method as stated in former study (Hossain et al., 2018). Replicability of the analysis was confirmed by testing at least ten times.

6.2.3. Lactate dehydrogenase (LDH) assay

The cell membrane damage was evaluated by lactate dehydrogenase activity kit (Promega, USA). Cells were treated with/without Hg^{2+} (5 μM), Zn^{2+} (100 μM), and combination of Hg^{2+} and Zn^{2+}-pre-exposure. After 48 h incubation, the cell culture medium was tested for LDH, as described by Hossain et al. (2018). The reproducibility was confirmed by executing this experiment four times.

6.2.4. Free sulfhydryl (SH) levels analysis

Intracellular glutathione levels or free sulfhydryl (SH) analysis was determined according to Hossain et al. (2018). PC12 cells were pretreated or treated with/without Zn^{2+} (100 μM) and Hg^{2+} (5 μM) for 48h, and then were washed with 1x PBS buffer. The protein extraction and

free sulfhydryl (SH) level determination were conducted as stated by Hossain et al. (2018). The assay was accomplished three times for explicability.

6.2.5. Extraction and Electrophoresis of DNA

Cells were exposed with/without Hg^{2+} (5 μM), Zn^{2+} (100 μM), and combination of Hg^{2+} and Zn^{2+}-pretreatment for 48 h. After that DNA extraction from the treated cells was operated by the process described by Hossain et al. (2018). Then the genomic DNA electrophoresis was assayed by agarose gel electrophoresis. 'Quantity one' software was utilized to measure the intensity of the electrophoresed DNA. Reliability of the analysis was confirmed through testing at least four times.

6.2.6. Flow cytometry analysis

Flow cytometry using Annxin V-FITC apoptosis detection kit (BioVision, USA) was conducted to evaluate the cell death process. With/without Hg^{2+} (5 μM), Zn^{2+} (100 μM), and combination of Hg^{2+} and Zn^{2+}-pretreatment, cells were incubated for 48 h. Then flow cytometry was done as stated in Hossain et al. (2020). The reliability of the result was confirmed by performing the experiment three times.

6.2.7. Western blot analysis

Cells were treated with/without Hg^{2+} (5 μM), Zn^{2+} (100 μM), and combination of Hg^{2+} and Zn^{2+}-pretreatment. After 48 h incubation protein extraction and electrophoresis were performed in line with the methods explained by Hossain et al. (2018). Polyclonal antibodies against mTOR (2972, Cell Signalling Technology), Heme oxygenase 1 (GTX101147, GeneTex), Nrf2 (Medical and Biological Laboratories Co., Japan), ERK1 (610031, BD Transduction Laboratories), phosphorylated akt (9267, CST), akt (4691, CST), p53 (2524, CST), Bcl2 (658701, Biolegend), Bax (2772, Cell Signalling Technology), Bcl-xL (612011, BD Transduction Laboratories), cleaved caspase-3 (NB100-56113SS, BioDynamics Laboratory Inc.), caspase-3 (GTX110543, GeneTEX), β-actin (GeneTEX), were used. Apoptosis Kit from Calbiochem® was used to check cytochrome c release. The reliability of the result was confirmed by performing the experiment three times.

6.2.8. Statistics

Data were displayed as the mean ± standard error of mean (SEM) of independent experiments (three experiments at least). The analysis was achieved using ANOVA, subsequently unpaired Student's t-test. Statistically significant was reflected at either $p<0.05$ or $p<0.01$ difference.

6.3. Results

6.3.1. Viability of the cells

PC12 cells were incubated with various concentration of Zn^{2+} (0-500 µM) for 48h to examine the effect of Zn^{2+} on the cells. Fig 6.1A showed the viability of cells exposed with Zn^{2+} concentration. Till 100 µM of concentration Zn did not showed any significant effect on the cells compared to the control group cells, which suggested that 100 µM of Zn is not toxic to PC12 cells. For this reason, 100 µM concentration was chosen to protect the cells from Hg induced toxicity.

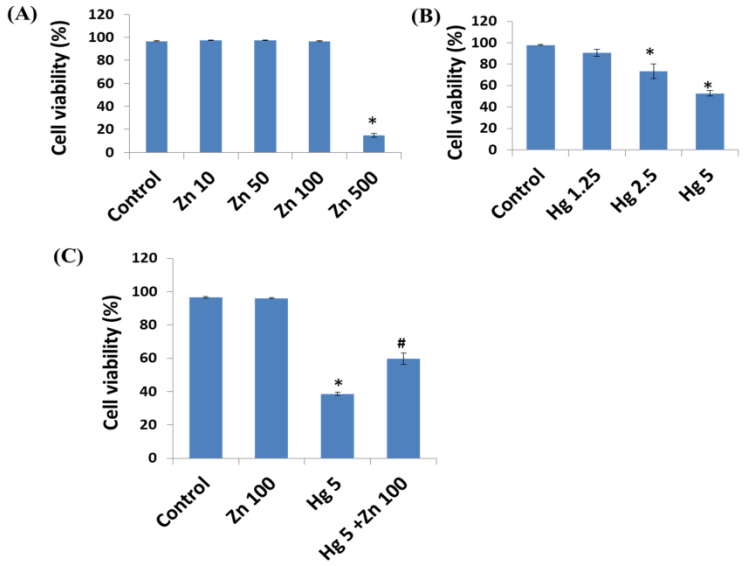

Figure 6.1: (A) Cell viability of PC12 cells cultured in medium with 0-500 µM of Zn, (B) Cell viability of PC12 cells cultured in medium with 0-5 µM of Hg, (C) Cell viability of PC12 cells pretreated with Zn (100 µM) and exposed to Hg (5 µM) for 48 h incubation. Error bars indicate mean ± SEM (n= at least 6). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only Hg-treated group.

To select the Hg^{2+} concentration for the combined treatment, PC12 cells were incubated with various concentrations of Hg. Fig. 6.1B presents the viability of cells incubated with 0-5 µM of Hg concentrations. Cell groups treated with 1.25 µM of Hg did not show any significant ($p<0.01$) change from the control group, although cells exposed to 2.5 and 5 µM of iHg, both revealed significant ($p<0.01$) cell viability decrease in comparison to the control group, with IC50 value of 4.37 (approximately 5). Hence, 5 µM of Hg was chosen to induce toxicity in cells to determine Zn-protection.

To evaluate the protection of Zn-pre-exposure against Hg mediated cytotoxicity, cells were exposed with/without Zn^{2+} (100 µM), Hg^{2+} (5 µM), and combination of Hg^{2+} and Zn^{2+}-

pretreatment for 48 h and cell viability assay was executed. Fig. 6.1C demonstrates the cell viability which was incubated with/without Hg^{2+} (5 µM), Zn^{2+} (100 µM) and combination of Hg^{2+} (5 µM) along with Zn^{2+} (100 µM)-pretreatment. As expected, after 48h of incubation the cells treated with Zn (100 µM)-treatment did not show any significant ($p<0.01$) variance, and cells exposed to Hg (5 µM) presented a significant ($p<0.01$) drop in the viability, in opposition to the control group (cells with no treatment). Interestingly, after 48h of incubation the cells exposed to Hg (5 µM) with Zn (100 µM)-pretreatment displayed a significant ($p<0.01$) improvement of viability, in opposition to the celss exposed to Hg (5 µM) alone. These results suggested that, Zn-pretreatment has a protective regulatory role against Hg-influenced toxicity.

6.3.2. Damaged membrane

Membrane integrity loss of the cell is an indication of cytotoxicity, which was examined firstly by trypan blue assay, and then by LDH activity assay. Fig 6.2A displayed the images of cells incubated with/without Hg^{2+} (5 µM), Zn^{2+} (100 µM), and combination of Hg^{2+} and Zn^{2+}-preexposure. After 48h cells were mixed with trypan blue. In this method, red and green stained cells are indicating membrane damaged cells and membrane intact cells, respectively. The cells incubated with only Hg, demonstrated a significantly high number of red colored (cell- membrane damaged or dead) cells, in comparison to control cells. Conversely, the cells pre-exposed to Zn (100 µM) beforehand Hg (5 µM) exposure, showed a substantial decrease of red colored cells, while compared to only Hg (5 µM)-treated cells.

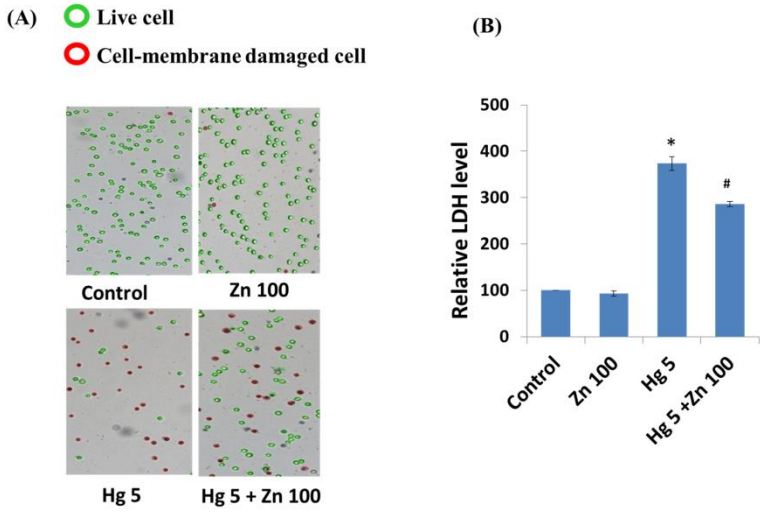

Figure 6.2: (A) Detection of cell wall damage in the cultured cells using trypan blue staining when cells were treated with/without Zn (100 µM), Hg (5 µM), or Hg (5 µM) along with Zn (100 µM)-pretreatment for 48 h. (B) LDH levels in the cultured medium for PC12 cells

treated with/without Zn (100 µM), Hg (5 µM), or Hg (5 µM) along with Zn (100 µM)-pretreatment for 48 h. Each column is shown as mean ± SEM, n=5. * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only Hg-treated group.

Cell-membrane damage was verified using LDH activity assay, another cytotoxicity valuation method. Fig 6.2B presents the LDH release of the cells treated or pretreated with/without Zn (100 µM) and Hg (5 µM) for 48h. The LDH release of the cells incubated with Hg (5 µM) alone, showed in a drastic rise ($p<0.01$) compared to that of the control group, suggesting a severe damage of the cell membrane. However, the LDH activity in media when cells pretreated with Zn (100 µM) followed by Hg (5 µM) exposure, revealed a significant ($p<0.05$) fall against the cells incubated with Hg (5 µM) alone; indicating amelioration in the cell-membrane. These findings, in cooperation with cell viability assay, supported that Zn-pretreatment protects cells from Hg induced cytotoxicity and membrane injury.

6.3.3. DNA degradation

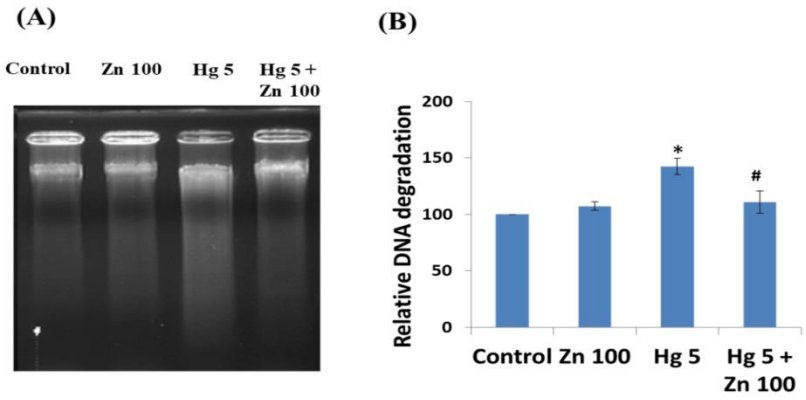

Figure 6.3: (A) Agarose gel electrophoresis of DNA isolated from PC12 cells treated with/without Zn (100 µM), Hg (5 µM), or Hg (5 µM) along with Zn (100 µM)-pretreatment for 48 h. Intensity of band obtained from DNA in the cells treated was shown below the electrophoresis band (B). Error bars are mean ± SEM (n=4). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only Hg-treated group.

DNA degradation assessment is another method for cytotoxicity assessment. Genomic DNA was extracted and electrophoresed, after the 48h incubation of the cells with/without Hg^{2+} (5 µM), Zn^{2+} (100 µM), and combination of Zn^{2+} (100 µM)-pretreatment and Hg^{2+} (5 µM). Fig 6.3A presents the image of electrophoresed DNA of the cells after the incubation and Fig.

6.3B showed the quantification of the DNA degradation intensity. The DNA degradation of the cells exposed to Hg (5 µM) alone displayed a significant increase as compared to that of the control group. Inversely, the cells pre-exposed to Zn (100 µM) beforehand Hg (5 µM)-exposure, demonstrated a significant ($p<0.05$) decrease in the DNA degradation amount, in compared to the cells incubated with Hg (5 µM) alone. These findings advised that Zn pretreatment before Hg-treatment protects the DNA from Hg stimulated DNA-degradation in cells.

6.3.4. Relative glutathione level

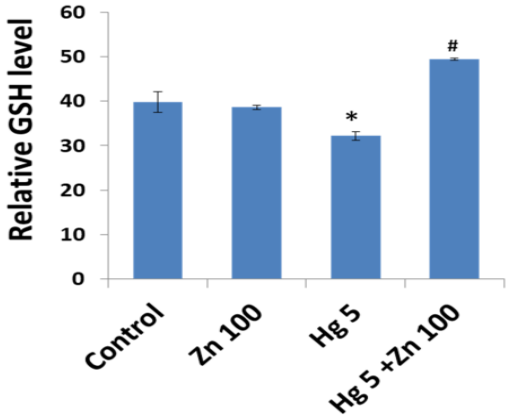

Figure 6.4: GSH contents in the cultured cells treated with/without Zn (100 µM), Hg (5 µM), or Hg (5 µM) along with Zn (100 µM)-pretreatment for 48 h. Error bars are mean ± SEM (n=3). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only Hg-treated group.

Glutathione level or free SH group is a dynamic controller for antioxidant defense mechanisms. Reduced glutathione (GSH) is reformed to its oxidized (GSSG) form for shielding the cells from ROS stimulated oxidative stress. In this study, cellular GSH level was assessed to understand the role of ROS in Hg-toxicity and the antioxidant defense status. Fig. 6.4 demonstrated the GSH level in cells exposed with/without Hg (5 µM) and Zn (100 µM), or with combination of Zn (100 µM)-pretreatment before Hg (5 µM)-exposure. The GSH level in the cells exposed to Hg (5 µM) alone was significantly ($p<0.01$) reduced in opposition to the control cells, suggesting a significant increase in ROS level in the cells. However, compared with the cells exposed to Hg (5 µM) alone, the GSH level of the cells exposed to Hg (5 µM) with Zn (100 µM)-pretreatment showed a significant ($p<0.01$) upsurge, representing significant drop in ROS amount in the cells. These outcomes indicated that Hg

encourages ROS mediated oxidative stress; whereas Zn defends from ROS facilitated oxidative stress and cytotoxicity by intensifying the GSH level.

6.3.5. Cell death detection

To know about the cell death mechanisms flow cytometry analysis was conducted. The cells exposed to Hg (5 µM) alone, revealed a drastic elevation ($p<0.05$) in apoptotic cells in opposition to the cells exposed to no treatment (Fig 6.5). On the other hand, the cells pre-exposed to Zn (100 µM) then exposed to Hg (5 µM), demonstrated a substantial decrease in apoptotic cells in opposition to the cells treated with iHg (5 µM) alone. These results indicated that Zn protected from Hg associated toxicity and suppressed apoptotic cell death.

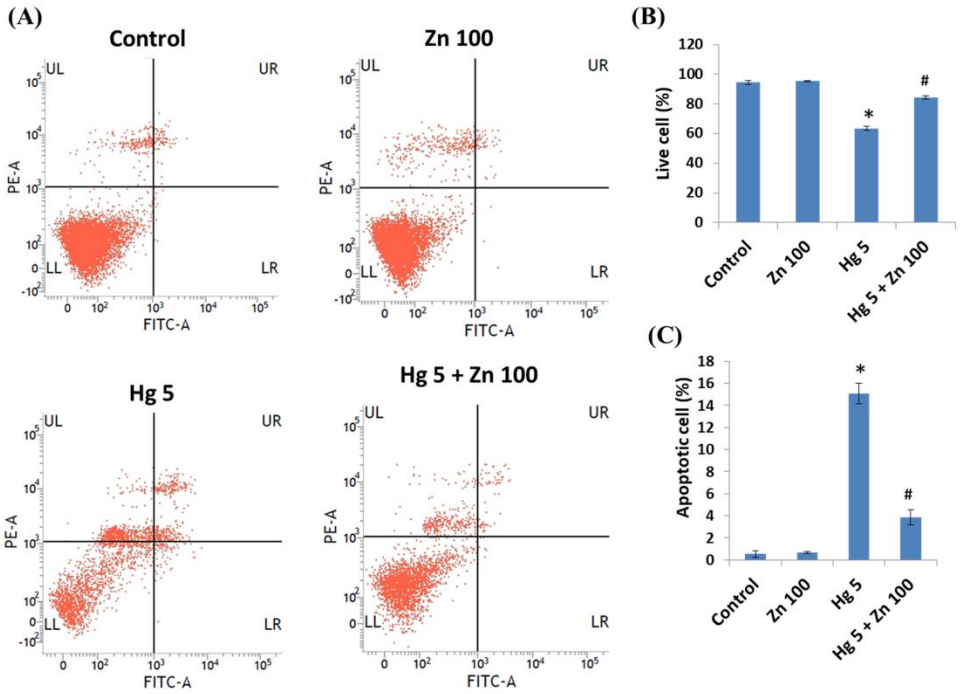

Figure 6.5: (A) Flow cytometry analysis of cultured PC12 cells, strained with Annexin V-FITC/PI after treatment with/without Zn (100 µM), Hg (5 µM), or Hg (5 µM) along with Zn (100 µM)-pretreatment for 48 h; and percentage of live cells (B) and apoptotic cells (C). Error bars are mean ± SEM (n=3). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only Hg-treated group.

6.3.6. Expression of the proteins

The molecular mechanisms responsible for Zn-pretreatment provided defense against Hg influenced cytotoxicity in cells, were investigated by western blotting analysis. The protein samples were extracted from the cells exposed with/without Hg (5 µM) and Zn (100 µM), or

combination of Hg (5 μM) with Zn (100 μM)-pretreatment; and western blotting was performed. The expressions of mTOR, akt, p-akt, Nrf2, ERK1, HO-1, p53, Bax, Bcl2, Bcl-xL, cytochrome c, caspase-3, cleaved caspase-3 and β-Actin were shown in fig 6.6 and 6.7.

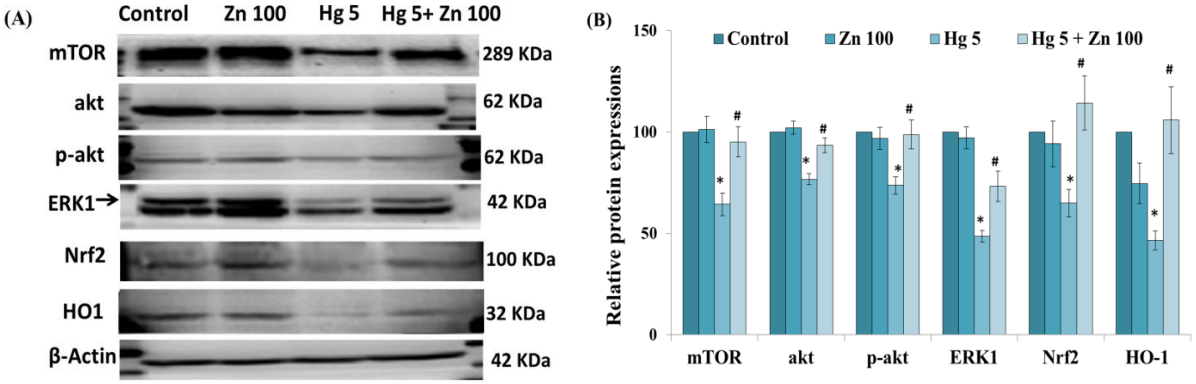

Figure 6.6: (A) Relative protein expression and (B) quantification of mTOR, akt, p-akt, ERK1, Nrf2, HO-1 and β-actin in the cultured cells treated with/without Zn (100 μM), Hg (5 μM), or Hg (5 μM) along with Zn (100 μM)-pretreatment for 48 h. Error bars are mean ± SEM (n= at least 4). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only Hg-treated group.

The mTOR protein plays an important governing role in cellular growth, proliferation, differentiation, survival and metabolism. And akt protein controls cellular survival, cell cycle, metabolism, and apoptosis by amending various downstream proteins, for example, NFkB, Nrf2, Bcl-2 family proteins etc. The expressions of mTOR, p-akt and akt were investigated in this study (fig 6.6). mTOR was considerably ($p<0.01$) decreased in cells treated with Hg (5 μM) alone, in contrast to the control. On the other hand, mTOR level was significantly ($p<0.01$) increased in the cells exposed with Hg (5 μM) and Zn (100 μM)-pretreatment, in opposition of cells exposed to Hg (5 μM) alone. Again, p-akt and akt of the cells exposed to Hg (5 μM) alone were significantly downregulated against the control cells; however both these protein expressions were significantly ($p<0.01$) reversed in cells exposed to Hg (5 μM) and Zn (100 μM)-pretreatment, compared to the cells exposed to Hg (5 μM) alone.

Mitogen-activated protein kinase 3 (MAPK3) or ERK1, is an enzyme that mediates miscellaneous biological functions depending on the cellular context, e.g., cell growth, adhesion, survival and differentiation via modulating transcription, translation and reorganizing cytoskeleton. The expression ERK1 was significantly ($p<0.01$) reduced in cells treated with Hg (5 μM) alone in comparison with the control cells; while ERK1 level was

significantly ($p<0.01$) surged in cells treated with Hg (5 µM) and Zn (100 µM)-pretreatment in comparison to the cells exposed to Hg (5 µM) alone (Fig 6.6).

Nrf2 is a crucial transcription factor which modulates a series of detoxifying and antioxidant defense gene expressions (for instance, HO1 regulation) to confer cytoprotection. To investigate about the involvement of nrf2-HO1 pathway, both of these protein expressions were investigated (Fig 6.6). The expression of nrf2 was ($p<0.01$) reduced significantly in cells treated with Hg alone, in contrast of the control group. Inversely, expression of nrf2 was significantly ($p<0.01$) augmented in cells exposed to Hg (5 µM) and Zn (100 µM)-pretreatment, while compared with the cells exposed to Hg (5 µM) alone. In addition, HO1 level was significantly ($p<0.01$) reduced in cells treated with Hg (5 µM) alone in comparison with the control cells; while HO1 level was significantly ($p<0.01$) increased in cells treated with Hg (5 µM) and Zn (100 µM)-pretreatment in comparison to the cells exposed to Hg (5 µM) alone (Fig 6.6).

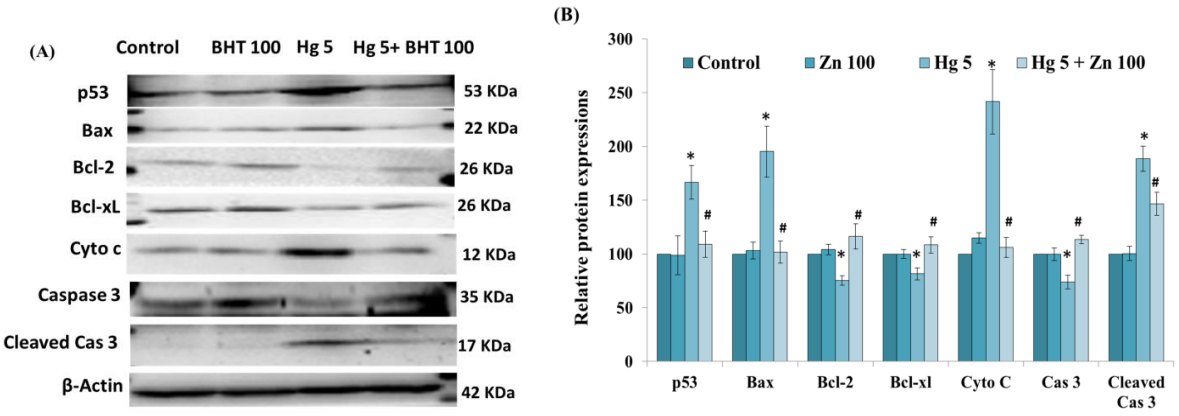

Figure 6.7: (A) Relative protein expression and (B) quantification of p53, Bax, Bcl-xL, Bcl-2, cytochrome c, caspase 3, cleaved caspase 3 and β-actin in the cultured cells treated with/without Zn (100 µM), Hg (5 µM), or Hg (5 µM) along with Zn (100 µM)-pretreatment for 48 h. Error bars are mean ± SEM (n= at least 4). * denotes $p < 0.05$, significantly difference from control group and # denotes $p < 0.05$, significantly difference from only iHg-treated group.

The p53 protein plays an important role in inducing several stress signals with a series of diverse anti-proliferative responses including activation of apoptosis. The p53 protein level was abruptly ($p<0.01$) elevated in cells incubated with only-Hg (5 µM) in opposition to the control cells, whereas p53 level was significantly ($p<0.05$) dropped in cells exposed to Hg (5 µM) and Zn (100 µM)-pretreatment as compared with cells incubated with only-Hg (5 µM) (Fig 6.7).

The Bcl-2 family involves members of pro-apoptotic or anti-apoptotic protein that either encourage or constrain apoptosis and regulate apoptosis. These proteins maintain the mitochondrial permeability and controls cytochrome c release into cytosol, which is a switch for intrinsic apoptosis. Here, Bcl-2 family proteins (anti-apoptotic Bcl2 and Bcl-xL, along with pro-apoptotic Bax) along with cytochrome c (cytosolic) release was studied (Fig 6.7). The Bcl-2 and Bcl-xL both were significantly ($p<0.05$) downregulated in cells exposed with Hg (5 µM) alone in opposition to the control cells. Contrarily, both proteins were significantly ($p<0.05$) upregulated when cell exposed to Hg (5 µM) and Zn (100 µM)-pretreatment as compared with the only-Hg (5 µM)-treated cells.

Additionally, the Bax level was ominously ($p<0.01$) increased in cells treated by Hg (5 µM) alone while compared with that of the control cells. Inversely, Bax level was significantly ($p<0.01$) reduced when cell treated with Hg (5 µM) and Zn (100 µM)-pretreatment in comparison to cells exposed to Hg (5 µM) only. Similarly, cytochrome c level in the cytosol was suggestively ($p<0.01$) upregulated in cells treated with only-Hg (5 µM) while compared with that of the control cells; and indicating intrinsic apoptosis occurred in only-Hg (5 µM) treated cells. Then again, cytochrome c level was significantly ($p<0.01$) downregulated when cell treated with Hg (5 µM) and Zn (100 µM)-pretreatment as compared with the only-Hg (5 µM)-treated cells; indicating prevention of intrinsic apoptosis by Zn (100 µM)-pretreatment.

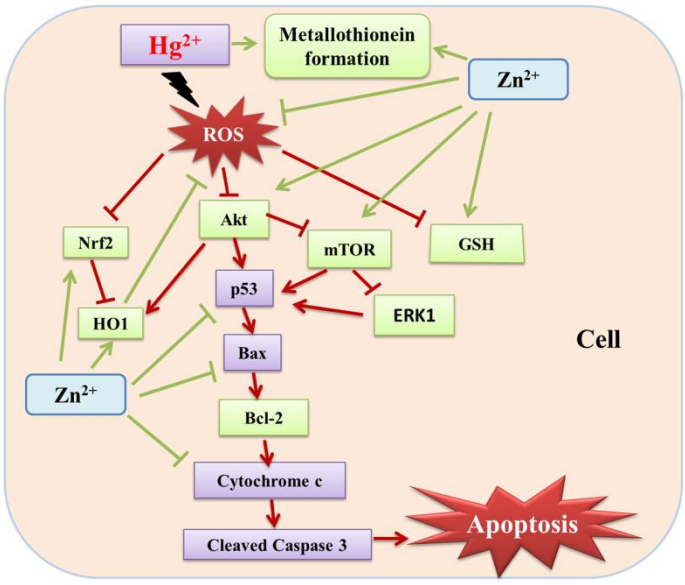

Figure 6.8: Schematic diagram of Zn-protection on Hg-induced cytotoxicity in PC12 cells.

A vital caspase family protein Caspase 3 and its activated form were also assayed (Fig. 6.7). Caspase 3 protein was significantly ($p<0.01$) decreased in Hg (5 µM)-exposed cells in opposition to the control cells; indicating caspase 3 activation in only-Hg (5 µM)-treated cells.

Again, caspase 3 level was significantly ($p<0.05$) rise in cells exposed to Hg (5 μM) and Zn (100 μM)-pretreatment, as compared with the only-Hg (5 μM)-treated cells. The activated caspase 3 level was drastically ($p<0.01$) upregulated in cells treated with Hg (5 μM)-only in opposition to the control group and confirmed apoptosis. Inversely, activated caspase 3 level was significantly ($p<0.05$) decreased in cells treated with Hg (5 μM) and Zn (100 μM)-pretreatment in comparison with the cells exposed to Hg (5 μM) alone and confirmed obstruction of apoptosis. Fig. 6.8 demonstrates a graphic diagram of Zn-pretreatment influenced protection in PC12 cells on Hg mediated cytotoxicity.

6.4. Discussion

Zn is a trace element required for the function of various enzymes and other cellular proteins, with antioxidant properties through various mechanisms, indicating an effective agent antagonizing Hg mediate toxicity. In this study, it was hypothesized that Zn has a curative role against inorganic Hg induced toxicity. The ameliorative role of Zn on Hg ($HgCl_2$)-mediated toxicity in PC12 cells was investigated, using several cellular toxicity assays. Here, it was apparent that Zn has protective effect on Hg-influenced toxicity. It was evident that in PC12 cells Zn prevents Hg-encouraged intrinsic apoptosis (Fig. 6.5) thru constraining LDH discharge (Fig. 6.2), lessening DNA destruction (Fig. 6.3), escalating GSH amount (Fig. 6.4), increasing response of akt, mTOR, ERK1, bcl2, bcl-xL, nrf2 and HO1 (Fig. 6.6); and repressing p53, bax, cytochrome c and cleaved caspase 3 (Fig. 6.7).

Hg is a toxic metal, induces toxicity in human, even in a low dose. In this study, Hg (5 μM) mediated toxicity in PC12 cells. Hg (5 μM)-exposed cells revealed a substantial viability lose (Fig 6.1), membrane injury (Fig 6.2), smeared DNA (Fig 6.3), glutathione attenuation (Fig 6.4). Similarly, inorganic Hg was reported to encourage cell viability loss, cell membrane damage (LDH release) and blocked DNA proliferation in murine T and B lymphoma cells (Kim et al., 2003). Also, inorganic Hg was reported to stimulate cell viability loss, ROS generation and GSH content decline in glioma cells (Lee et al., 2001). In my previous research, inorganic Hg motivated apoptosis type of programmed cell death in PC12 cells (Hossain et al., 2020). Consistently, in the present research inorganic Hg stimulated apoptosis in PC12 cells, in intrinsic pathway (Fig 6.5, Fig 6.6 and Fig 6.7). Hg triggered suppression of mTOR, akt, p-akt, Erk1, nrf2, HO1, bcl-2, and bcl-xL expressions; along with stimulating the expressions of P53, bax, cleaved caspase 3 and cytochrome c (Fig 6.6 and Fig 6.7) interactively, indicating oxidative stress and ROS production in PC12 cells. Previous reports also reported inorganic Hg induced oxidative stress and apoptosis (Kim et al., 2003; Teixeira

et al., 2018). It was demonstrated here that Hg (5 μM) persuades toxicity and cell death in PC12 cells by means of negotiating cellular antioxidant defense accompanying with oxidative stress (Fig 6.8).

Oxidative stress is a metabolic dysfunction that results in the oxidation of biomolecules, causes oxidative damage of cells and tissues; and consequently contributes to several chronic diseases. Certainly, Zn is one of the most significant minerals for health, for its antioxidant properties; and therefore, is potential to protect against oxidative stress. Zn involves in the proper functioning of the antioxidant defense system by working as a co-factor for important enzymes. Moreover, Zn also persuades metallothioneins (MT) synthesis. MTs are proteins that reduce hydroxyl radicals and sequester ROS in stressful situations. Moreover, antioxidant enzymes (such as GPx and glucose-6-phosphate dehydrogenase) restoration, increased amount of vitamin C, vitamin E and GSH were considered as Zn induced protection mechanisms, apart from formation of MT (Fukino et al., 1984). Numerous researchers confirmed about Zn-protection against oxidative stress in several diseases (Marreiro et al., 2017). Jadan-Piedra et al. (2018) stated that Zn-induced amelioration against Hg^{2+} majorly via MT generation and antioxidant activities. In addition, concomitant MT formation, declined oxidative stress, with prevention of Hg accumulation have been recognized to be Zn-induced defense mechanisms counter to Hg-toxicity (precisely inorganic Hg toxicity) (Rahman et al., 2019).

There are several reports on Zn-protection against metal induced oxidative stress and toxicity reduction. For instance, protection of Zn against lead-induced oxidative stress and toxicity in Wistar rats (Anjum et al., 2017), on cadmium-mediated cytotoxicity in PC12 cells (Rahman et al., 2017), and on arsenic induced toxicity with MT formation in rat (Ganger et al., 2016), have been reported. Moreover, several in vivo studied reported Zn-protection against Hg mediated toxicity. Franciscato et al. (2011) reported that delayed biochemical alterations caused by Hg-intoxication prevented by Zn pre-exposure. Mesquita et al. (2016) demonstrated Zn-protection on Hg toxicity in female rats. However, the interaction of Zn with Hg is less investigated, with no exploration in cellular level. Further study is required to assess the inhibitory effect of Zn in Hg mediated toxicity in both cellular and molecular level.

Our present study demonstrated a couple of key roles of Zn (100 μM)-amelioration against Hg (5 μM)-mediated cytotoxicity in PC12 cells in pre-exposure. Here, it was observed that Zn impeded Hg mediated toxicity in PC12 cells by elevating cell survival, enhancing intact cellular membrane, limiting DNA damage, improving cellular glutathione amount, upsurging expressions of mTOR, akt, ERK1, nrf2, HO1, bcl2 and bcl-xL, and shrinking pro-apoptotic

p53, bax, cytochrome c and cleaved caspase 3; and finally seizing apoptosis. These protection provided by Zn is expected to be resulted from the antioxidant properties of Zn. Previously, Zn treatment was reported to protect cells from cadmium induced cytotoxicity through rising the viability and keeping membrane intact (decreasing LDH discharge) by rahman et al. (2017). Kumar et al. (2010) informed that Zn-administration to arsenic-treated rats significantly increased GSH amount compared to arsenic-exposed rats. Again, Szuster-Ciesielska et al. (2000) reported that Zn protected HeLa cells from cadmium mediated DNA degradation and apoptosis. Also, similar to this study, Zn protected PC12 cells from cadmium-toxicity by maintaining mitochondrial permeability intact (increasing bcl2, and decreasing bax and cytochrome c release) (Rahman et al., 2017). Even Nrf2 activation is a potential protective mechanism of Zn for toxicity reduction. Similar to this study, Cortese et al. (2008) proved that endothelial cells were protected from hydrogen peroxide by Zn via stimulation of Nrf2, HO1 and GSH synthesis. Similarly, Ha et al. (2006) stated that Zn significantly increased GSH levels through activation of the ARE-Nrf2 pathway in ARPE-19 cells. Maremanda et al. (2014) Shown that Zn supplementation protected against cyclophosphamide-induced testicular damages in rat by modulating metallothionein, tesmin and Nrf2 associated pathways. Similar to these reports, present results indicated a significant role of nrf2-HO1-pathway in Zn-pretreatment provided protection. For the first time in this research it was indicated to be involved in Zn-pretreatment against inorganic Hg influenced cytotoxicity in PC12 cells, though additional assessment is needed to expose the details of nrf2-HO1 pathway in PC12 cells, along with other cell lines. Moreover, together with nrf2, glutathione amount increase by Zn-pretreatment contributed in defending cells against inorganic Hg-induced oxidative stress and toxicity. Fig. 6.8 demonstrates a graphic diagram of Zn-pretreatment influenced protection against Hg mediated cytotoxicity in PC12 cells via its antioxidant properties. Extensive investigation for involvement of metallothionein in Zn-pretreatment to inhibit Hg-cytotoxicity is required for better understanding.

6.5. Conclusion

Inorganic Hg induces cytotoxicity in PC12 cell, along with intrinsic apoptotic cell death. Zn pretreatment is partially effective in reversing inorganic Hg induced toxicity. Zn-pretreatment can protect cells from inorganic Hg induced cytotoxicity and intrinsic apoptosis via its antioxidant properties, especially through glutathione increase and nrf2-mediated pathway. Research findings from this study could be applicable in future researches of Zn-protection and its therapeutic use against heavy metal induced toxicity and diseases in human.

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Chapter 7: General conclusion

Present research carried out with some particular and precise objectives majorly related to environmental toxic metals and their deadly effects in biological systems along with remediation perspectives. Environmental pollution mainly caused by excess population, unplanned urbanization and industrialization. To achieve the economic growth the necessity for a cleaner environment is always overlooked and results in a polluted environment with environmental diseases affecting heath. Developing countries (for example, Bangladesh) are more vulnerable to environmental pollutions than the developed countries, where rapid growth of economy is prioritized over quality of environment and public health issues. As a result all components of environment (air, water, soil) are loaded with industrial chemicals, such as toxic heavy metals, dyes, pesticide, herbicides, persistent organic pollutants, particulate matters etc; which in turn enter into food chain. Recently polluting chemicals, especially heavy metals have been found in foods (rice, vegetables, fishes, chickens, milk, and fruits), drinking water, and other organisms, indicating severe pollution and health hazard. Better understanding about the molecular mechanism/s of toxic metals in biological system and searching for their therapeutics to reduce their health effects is crucial. Numerous reports have been published about various food components (Se, Zn, DHLA and BHT) and their therapeutic potential as antioxidants against toxicants in cellular lever as well as in animal models. Therefore, to elucidate the protective effects of Se, DHLA, BHT and Zn against Cd and Hg toxicity, this research was carried out in cellular model to explore the biomolecular mechanisms of potential protection. Among the protective antioxidants, Se and Zn are essential trace elements, DHLA is a popular dietary supplement, and BHT is a popular food additive with antioxidant properties. Se exerts antioxidant properties via scavenging the reactive oxygen species, producing other antioxidants (such as GPx), can form metabolic complexes to detoxify metals and can boost up antioxidant defense system. The antioxidant properties exerted by Zn is via direct ROS scavenging, formation of MT, modulating the uptake and bioavailability of metals by contributing in Zn-transporter and other metal transporters, and modulating glutamate-cysteine ligase in glutathione synthesis. DHLA exerts antioxidant properties through scavenging ROS, metal chelation, endogenous antioxidant (glutathione, vitamin C and E) regeneration and modulation of various cellular pathways. BHT employs antioxidant activity as a strong scavenger of ROS.

The mechanism/s of cyto-protection by Se against Cd-induced cytotoxicity using PC12 cells was investigated in chapter 2. Moreover, the Se induced toxicity was investigated together

with Cd induced toxicity. Cytotoxicity assays showed that higher Se (≥10 µM) concentrations induce toxicity in PC12 cells. Western blot analyses confirmed that Se (≥10 µM) promotes autophagic cell death via inhibition of mTOR activation and p62 accumulation due to increase of cellular oxidative stress. Se (≥10 µM) also exerted cell viability decrease, membrane damage, DNA degradation with glutathione decrease. However, lower concentration of Se (5 µM) protected the cells from Cd (5 µM)-induced toxicity. Co-exposure of non-toxic Se (5 µM) and toxic Cd (5 µM) showed to increase cell viability, glutathione and glutathione peroxidase 1 (GPx1) levels, and to decrease DNA fragmentation and lactate dehydrogenase (LDH) activity compared to Cd-treated (5 µM) cells alone. In addition, western blot analyses indicated that Cd induces intrinsic apoptotic cell death in PC12 cells via ERK1 downregulation and cytochrome c release. Interestingly, the co-exposure of Se with Cd significantly altered all toxic effects, such as, decreased the release of cytochrome c into cytosol from mitochondria, and up-regulated ERK1 protein to inhibit Cd-induced apoptosis. From this study it can be suggested that, Se (≥10 µM) possess cytotoxicity in PC12 cells; and co-presence of Se (5 µM) with Cd (5 µM) protects against Cd-induced apoptosis in PC12 cells due to inhibition of Cd-induced oxidative stress and subsequently suppression of mitochondrial apoptosis pathway (Fig 7.1). These outcomes could be useful for eradicating Cd-toxicity by supplementing Se as therapeutics.

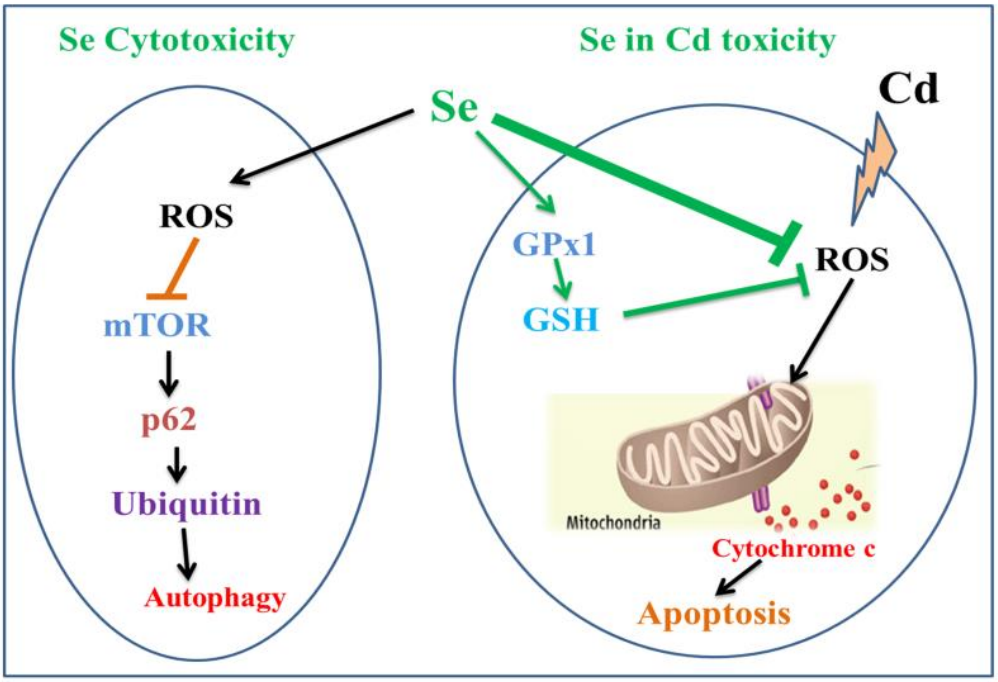

Figure 7.1: Biomolecular mechanisms of Se toxicity, and Se-protection against Cd-mediated toxicity in PC12 cells.

In Chapter 3, the regulatory effects of DHLA against inorganic Hg mediated cytotoxicity was demonstrated in PC12 cells. Hg is an extremely dangerous environmental contaminant, whereas DHLA is a potent antioxidant. In this study, the mechanism/s of cytotoxicity of Hg was investigated, as well as, the cyto-protection of DHLA against Hg induced toxicity. Treatment of cells with Hg of various concentrations (0-2.5 µM) for 48 h caused drastic toxic effects, such as, cell viability loss, high level of lactate dehydrogenase (LDH) release, DNA damage, cellular glutathione (GSH) level decrease and increased Hg accumulation. Moreover, protein level expressions of akt, p-akt, mTOR, GR, NFkB, ERK1, Nrf2 and HO-1 in cells were down regulated; and cleaved caspase 3 and cytochrome c release were upregulated after Hg (2.5 µM) exposure and thus indicating apoptosis induction. However, pretreatment with DHLA (50 µM) for 3 h before Hg (2.5 µM) exposure showed reversing the Hg-induced cytotoxicity by inhibiting cell viability loss, LDH release, DNA damage, GSH decrease and limiting Hg accumulation. In addition, DHLA pretreatment positively regulated the protein expressions of akt, p-akt, mTOR, GR, NFkB, ERK1, Nrf2, HO-1, cleaved caspase 3 and cytochrome c. To conclude it can be said that, DHLA could attenuate Hg-induced cytotoxicity via limiting Hg accumulation (which indicates chelate formation), boosting up of antioxidant defense and inhibition of apoptosis in cells (Fig 7.2).

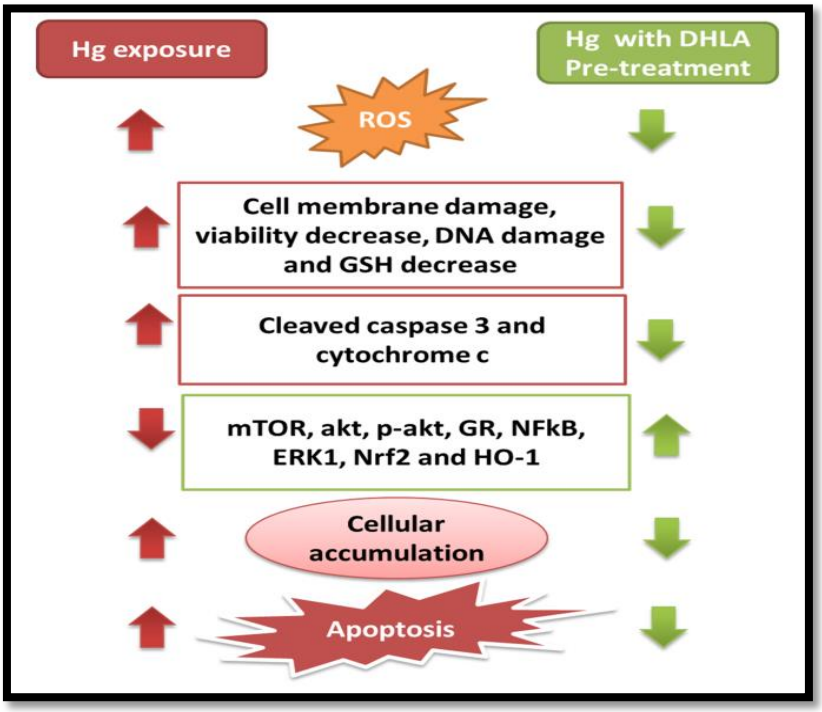

Figure 7.2: Biomolecular mechanisms of DHLA-pretreatment against Hg-mediated toxicity in PC12 cells.

In chapter 4, Se-protection against inorganic Hg-induced cytotoxicity was presented in PC12 cells using co-exposure. Cytotoxic assays and western blotting analysis have been demonstrated that Hg (5 µM) induced oxidative stress and intrinsic apoptosis *via* oxidizing glutathione, damaging DNA, degrading cell membrane integrity, down-regulating mTOR, p-mTOR, akt, ERK1 and caspase 3, and up-regulating cleaved caspase 3 and cytochrome c release in PC12 cells 48 h after incubation. However, co-exposure of Se (5 µM) appeared to inhibit intrinsic apoptosis and oxidative stress induced by Hg *via* boosting GPx contents, improving reduced form glutathione contents, limiting DNA degradation, improving cell membrane integrity, up-regulating mTOR, p-mTOR, akt, ERK1 and caspase 3, and down-regulating cleaved caspase 3 and cytochrome c leakage in PC12 cells. Moreover, flow cytometry analysis confirmed that co-treatment of Se with iHg protected PC12 cells from iHg mediated apoptotic cell death. Thus it can be concluded that, glutathione level decrease plays a significant role in Hg induced oxidative stress, and co-treatment of Se attenuates Hg-cytotoxicity through its antioxidant properties by influencing GSH and GPx content (Fig 7.3).

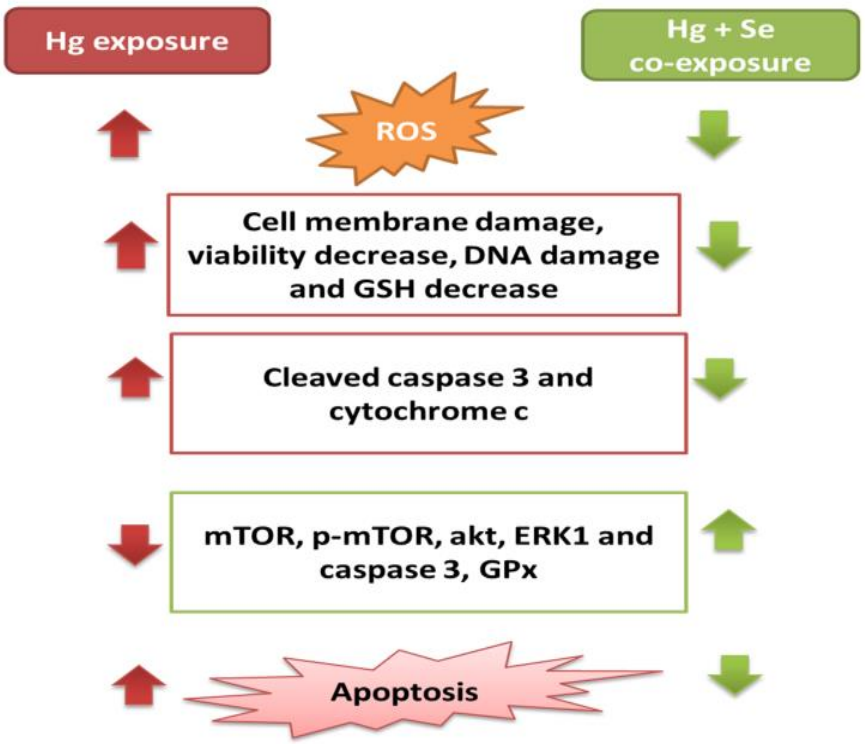

Figure 7.3: Biomolecular mechanisms of DHLA-pretreatment against Hg-mediated toxicity in PC12 cells.

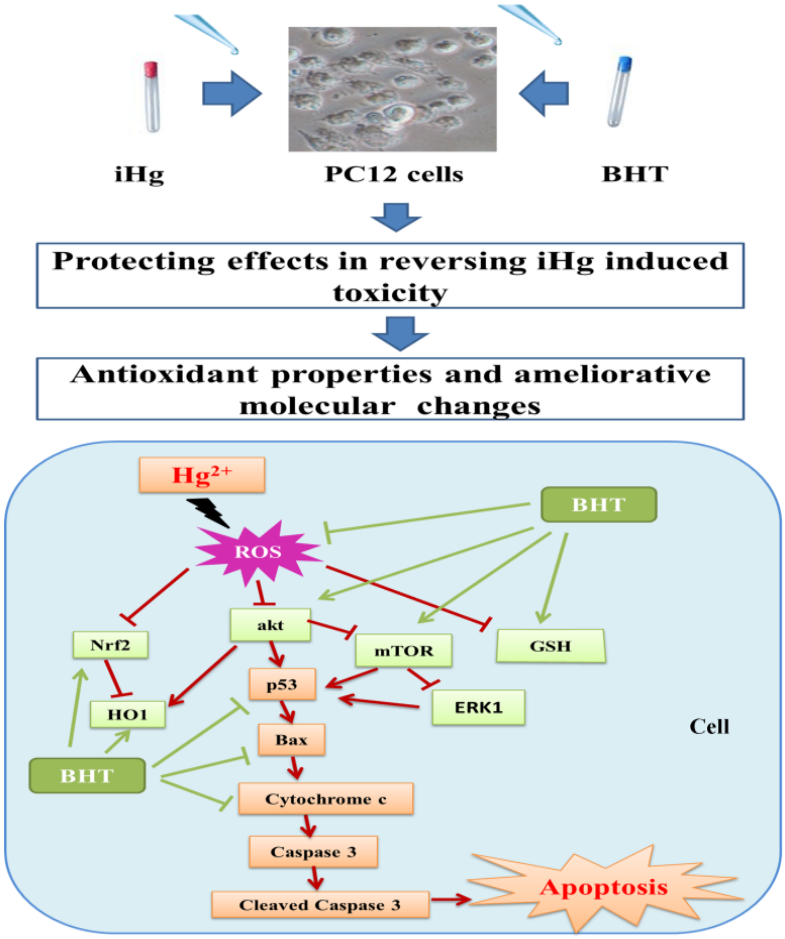

Figure 7.4: Biomolecular mechanisms of BHT-protection against Hg-mediated toxicity in PC12 cells.

Chapter 5 showed modulating effects of BHT on inorganic Hg induced cytotoxicity and underlying molecular mechanisms. BHT (100 µM) and Hg (5 µM) co-exposure showed prevention against Hg-influenced toxicity by improving cell viability, cell membrane damage, DNA damage and oxidative stress. Flow cytometry analysis depicted that BHT co-treatment with iHg was successfully reversed apoptosis cell death imposed by iHg alone in PC12 cells. In addition, BHT co-treatment reversed the expressions of cytoprotective proteins and apoptotic proteins. The western blotting analysis showed a significant downregulation of pro-apoptotic proteins, such as p53, bax, cytochrome c, cleaved caspase 3 and a significant upregulation of anti-apoptotic protein bcl-xL. Moreover, BHT co-treatment restored the expressions of mTOR, akt, p-akt, ERK1, nrf2 and HO1 pro-survival proteins, from iHg-induced suppressions in that of protein expressions. These results indicated that BHT inhibits Hg-mediated toxicity through enhancing antioxidant defense, and by preserving mitochondrial membrane integrity (Fig. 7.4).

In chapter 6, the ameliorative actions of Zn-pretreatment on inorganic Hg induced cytotoxicity and its underlying mechanisms of action using PC12 cells were reported. Cells pretreated with Zn (100 μM) before Hg (5 μM)-exposure showed a significant improvement in cell viability, cell membrane, DNA damage, glutathione level and apoptotic cells, with activation of nrf2-HO1 pathway and keeping mitochondrial membrane integrity, indicating inhibition of intrinsic apoptosis by Zn-pretreatment. Western blot analysis showed a significant downregulation of pro-apoptotic proteins, such as p53, bax, cytochrome c, cleaved caspase 3 and a significant upregulation of anti-apoptotic protein bcl-xL and bcl-2. Moreover, Zn pre-treatment restored the expressions of mTOR, akt, p-akt, ERK1, nrf2 and HO1 pro-survival proteins, from iHg-induced suppressions in that of protein expressions. Furthermore, flow cytometry analysis confirmed that pre-treatment of Zn protected PC12 cells from iHg mediated apoptotic cell death. Thus suggested that Zn-pretreatment as an antioxidant not only improves glutathione content but also induces activation of nrf2-HO1 pathway, which would tend to suppress Hg-cytotoxicity and inhibit intrinsic apoptosis (Fig 7.5).

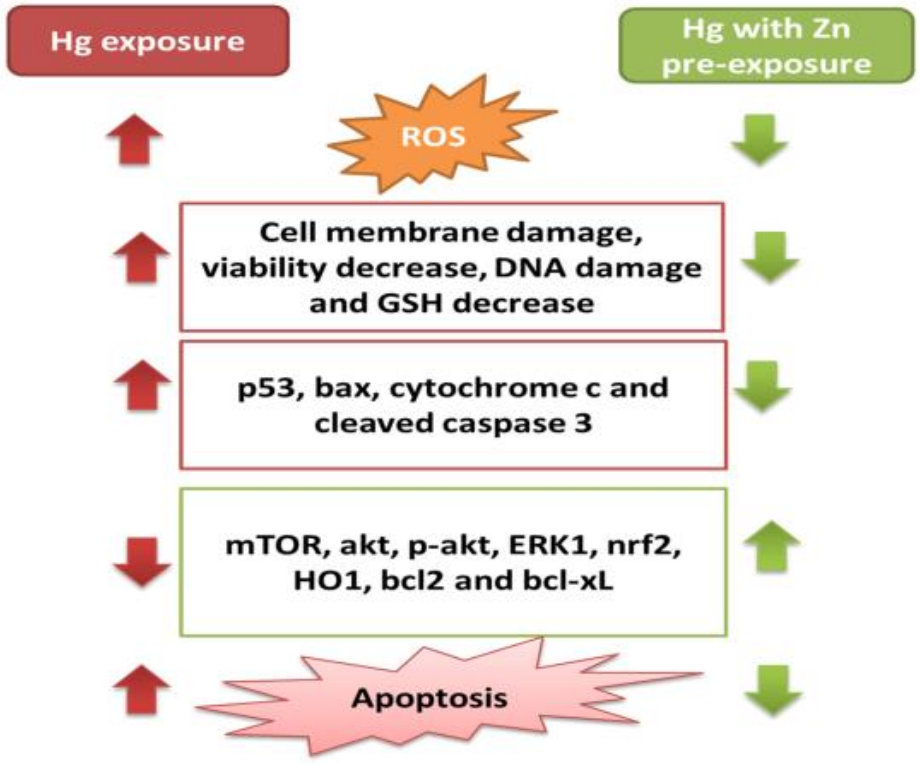

Figure 7.5: Biomolecular mechanisms of Zn-protection against Hg-mediated toxicity in PC12 cells.

Thus, finally it can be concluded that Se demonstrated ameliorative actions against Cd induced toxicity; and Se, DHLA, BHT and Zn, all of them showed beneficial role against Hg mediated toxicity in PC12 cells. Therefore, these dietary components could be perfect therapeutics to combat metal induced harmful effects in biological systems. Further *in vivo* experiments are required to assess the affectivity of these food components (Se, DHLA, BHT and Zn) against toxic metal exposure in human. In conclusion, these food components could be very auspicious to defend human body from toxic heavy metals.